

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/573, C12N 9/00, C07K 16/00, C07H 21/04</b>	<b>AI</b>	<b>(11) International Publication Number:</b> <b>WO 97/37225</b> <b>(43) International Publication Date:</b> 9 October 1997 (09.10.97)
<b>(21) International Application Number:</b> PCT/US97/05198 <b>(22) International Filing Date:</b> 28 March 1997 (28.03.97)  <b>(30) Priority Data:</b> 60/015,385 2 April 1996 (02.04.96) US 08/790,186 29 January 1997 (29.01.97) US  <b>(71) Applicant:</b> BLOOD CENTER RESEARCH FOUNDATION [US/US]; 8727 Watertown Plank Road, Milwaukee, WI 53226-3548 (US).  <b>(72) Inventors:</b> WIEDMER, Therese; 6815 West Mendota Court, Mequon, WI 53092 (US). SIMS, Peter, J.; 6815 West Mendota Court, Mequon, WI 53092 (US).  <b>(74) Agent:</b> BAKER, Jean; Quarles & Brady, Suite 2550, 411 East Wisconsin Avenue, Milwaukee, WI 53202 (US).	<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> A PROTEIN PREPARATION THAT MEDIATES Ca <sup>2+</sup> -DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF  <b>(57) Abstract</b>  A protein preparation that mediates Ca <sup>2+</sup> -dependent transbilayer movement of phospholipids is disclosed. A recombinantly engineered DNA sequence encoding the protein, an inhibitor of the protein activity, genetically engineered cells with altered protein activity, and therapeutic methods are also disclosed.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

A PROTEIN PREPARATION THAT  
MEDIATES  $\text{Ca}^{2+}$ -DEPENDENT TRANSBILAYER MOVEMENT OF  
PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

United States Government may have commercial rights under Grant R01 HL36946 from Heart, Lung, & Blood Institute, National Institutes of Health.

BACKGROUND OF THE INVENTION

5       The exposure of phosphatidylserine (PS) and other  
aminophospholipids (aminopL) on the surface of activated or  
injured blood cells and endothelium is thought to play a key  
role in the initiation and regulation of blood coagulation.  
De novo surface exposure of aminophospholipids has also been  
10   implicated in the activation of both complement and  
coagulation systems after tissue injury, and in removal of  
injured or apoptotic cells by the reticuloendothelial system.  
Although migration of these phospholipids (PL) from  
inner-to-outer plasma membrane leaflets is known to be  
15   triggered by elevated intracellular  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_i$ ) and to be  
associated with vesicular blebbing of the cell surface, little  
is known about the cellular constituents that participate in  
this process.

Role of cell surface PS in coagulation. Several enzyme  
20   complexes of the coagulation cascade require assembly on a  
receptive membrane surface for full expression of catalytic  
activity (K. G. Mann, et al., *Annu. Rev. Biochem.* 57:915-956,  
1988; S. Krishnaswamy, et al., *J. Biol. Chem.* 267:26110-26120,  
1992; P. B. Tracy, *Semin. Thromb. Hemost.* 14:227-233, 1988).  
25   In the case of the tenase (FVIIIaFIXa) and prothrombinase  
(FVaFXa) complexes, this surface catalytic function of the  
plasma membrane is not normally expressed by quiescent cells,  
but is rapidly induced upon cell activation (in platelets) or  
upon cell injury (in platelets, endothelium and other cells)  
30   (E. M. Bevers, et al., *Blood Rev.* 5:146-154, 1991; J. Rosing,  
et al., *Blood* 65:319-332, 1985; E. M. Bevers, et al., *Eur. J.*  
*Biochem.* 122:429-436, 1982; E. M. Bevers, et al., *Biochim.*

Biophys. Acta 736:57-66, 1983; T. Wiedmer, et al., Blood 68:875-880, 1986). Although specific cell surface protein receptors for FVa and FVIIIa have been postulated, these factors show specific avidity for PS-containing liposomes, and in cell-free systems, this lipid alone can support the catalytic function of the prothrombinase and tenase enzymes (J. Rosing, et al., supra, 1985; M. E. Jones, et al., Thromb. Res. 39:711-724, 1985; G. E. Gilbert, et al., Biochemistry 32:9577-9585, 1993; G. E. Gilbert, et al., J. Biol. Chem. 265:815-822, 1990; G. E. Gilbert, et al., J. Biol. Chem. 267:15861-15868, 1992). We and others have shown that PS rapidly moves to the surface of the plasma membrane upon platelet stimulation, and that this exposure of PS correlates with expression of the platelet's FVa & FVIIIa binding sites and expression of surface catalytic function for tenase and prothrombinase (P. Williamson, et al., Biochemistry 31:6355-6360, 1992; F. Bassé, et al., Biochemistry 32:2337-2344, 1993; C.-P. Chang, et al., J. Biol. Chem. 268:7171-7178, 1993; J. Connor, et al., Biochim. Biophys. Acta 1025:82-86, 1990; P. Comfurius, et al., Biochim. Biophys. Acta 1026:153-160, 1990). Smeets, et al., Biochim. Biophys. Acta 1195:281-286, 1994; Williamson, et al., Biochem. 34:10448-10455, 1995; Bratton D.L., J. Biol. Chem. 269:22517-22523, 1994). Additional evidence that surface-exposed PS provides the physiological receptor site for these enzyme complexes is provided by (1) the capacity of PS-containing liposomes or phosphoserine to compete binding of FVIIIa to activated platelets (G.E. Gilbert, et al., J. Biol. Chem. 266:17261-17268, 1991), (2) the capacity of annexin V and other proteins with affinity for membrane PS to mask the FVa and FVIIIa binding sites expressed by activated platelets (P. Thiagarajan, et al., J. Biol. Chem. 265:17420-17423, 1990; P. Thiagarajan, et al., J. Biol. Chem. 266:24302-24307, 1991; J. Dachary-Prigent, et al., Blood 81:2554-2565, 1993; J. Sun, et al., Thromb. Res. 69:289-296, 1993); (3) evidence that platelets congenitally deficient in inducible FVa and FVIIIa receptors are also defective in stimulated exposure of plasma membrane PS ("Scott syndrome"; see below) (J. P. Miletich, et

- al., *Blood* 54:1015-1022, 1979; J. Rosing, et al., *Blood* 65:1557-1561, 1985; P. J. Sims, et al., *J. Biol. Chem.* 264:17049-17057, 1989; S. S. Ahmad, et al., *J. Clin. Invest.* 84:824-828, 1989; F. Toti, et al., *Blood* 87:1409-1415, 1996).
- 5 In addition to the catalytic function PS provides to the prothrombinase and tenase complexes, surface exposed aminophospholipids have been shown to promote the activities of the tissue factor-FVIIa and protein S-activated protein C enzyme complexes of the coagulation system, as well as the
- 10 activity of the alternative pathway C3-convertase (C3bBb enzyme complex) of the complement system (W. Ruf, et al., *J. Cell. Biol.* 266:2158-2166, 1991; F. J. Walker, *J. Biol. Chem.* 256:11128-11131, 1981; R. H. Wang, et al., *J. Clin. Invest.* 92:1326-1335, 1993; P. F. Neuenschwander, et al., *Biochemistry*
- 15 34:13988-13993, 1995).

- In addition to the central role that inducible expression of plasma membrane PS is thought to play in the platelet hemostatic response, the surface exposure of PS and phosphatidylethanolamine (PE) in response to membrane injury
- 20 has been implicated in a variety of thrombotic and inflammatory disorders. For example, repeatedly sickled SS hemoglobin erythrocytes exhibit increased surface exposure of PS, which promotes prothrombinase assembly and accelerates plasma clotting *in vitro*, and may contribute to thrombotic
- 25 complications that can arise in sickle cell disease (P. F. Franck, et al., *J. Clin. Invest.* 75:183-190, 1985; N. Blumenfeld, et al., *Blood* 77:849-854, 1991). Increased PE exposure on sickled RBCs (and other cells) has also been shown to promote complement activation with resulting accumulation
- 30 of C3b/C3d and C5b-9 on the cell surface, potential factors contributing to the accelerated clearance and increased fragility of these cells (R. H. Wang, et al., *supra*, 1993). PS exposure secondary to immune injury to the endothelium has also been implicated in the thrombo-embolic complications of
- 35 hyperacute graft rejection, and PS exposure secondary to C5b-9 accumulation on platelets and red cells has been suggested to contribute to the high risk of venous thrombosis in Paroxysmal Nocturnal Hemoglobinuria (J. L. Platt, et al., *Immunol. Today*

11:450-6; discuss, 1990; A. P. Dalmasso, *Immunopharmacology* 24:149-160, 1992; A. P. Dalmasso, et al., *Am. J. Pathol.* 140:1157-1166, 1992; T. Wiedmer, et al., *Blood* 82:1192-1196, 1993; K. K. Hamilton, et al., *J. Biol. Chem.* 265:3803-3814, 1990; S. P. Kennedy, et al., *Transplantation* 57:1494-1501, 1994)). In the "antiphospholipid syndromes," the interaction of exposed plasma membrane PS and PE with plasma proteins is now generally believed to induce offending antigens (M.D. Smirnov, et al., *J. Clin. Invest.* 95: 309-316, 1995).

10        Relationship of PS exposure to programmed cell death.

Programmed cell death (apoptosis) is now recognized to be central to the selective elimination of mammalian cells during embryogenesis, tissue re-modeling, and in the clonal selection of immune cells (P. D. Allen, et al., *Blood Rev.* 7:63-73, 1993; J. J. Cohen, *Immunol. Today* 14:126-130, 1993). The apoptotic cell undergoes characteristic changes, including elevated  $[Ca^{2+}]_i$ , altered phospholipid packing, surface exposure of PS, plasma membrane blebbing and vesiculation, cell shrinkage, chromatin condensation, nucleolar desintegration, and at late stages, DNA degradation by  $Ca^{2+}/Mg^{2+}$ -dependent endonuclease(s), with characteristic fragmentation into 180 bp multimers ("DNA laddering"). The transcriptional events that initiate apoptosis remain unresolved, but evidence implicates certain proto-oncogenes, including c-myc as activators, and other proto-oncogenes, including bcl-2, as suppressors (P. D. Allen, et al., *supra*, 1993; J. C. Reed, *J. Cell. Biol.* 124:1-6, 1994). In thymocytes and B-lymphocytes, an apoptotic transformation can be induced by dexamethasone (activating glucocorticoid receptors) and by cAMP (protein kinase A pathway) (D. J. McConkey, et al., *J. Immunol.* 145:1227-1230, 1990; N. Kaiser, et al., *Proc. Natl. Acad. Sci. USA* 74:638-642, 1977; J. J. Cohen, et al., *J. Immunol.* 132:38-42, 1984; R. Merino, et al., *EMBO J.* 13:683-691, 1994; M. K. Newell, et al., *Proc. Natl. Acad. Sci. USA* 90:10459-10463, 1993), as well as directly through treatment with  $Ca^{2+}$  ionophore (Z.-Q. Ning, et al., *Eur. J. Immunol.* 23:3369-3372, 1993), implicating  $[Ca^{2+}]_i$  as a

central mediator of the cellular changes that accompany apoptosis. The similarity of the plasma membrane changes noted for apoptotic cells, to those elicited by elevation of  $[Ca^{2+}]$ , in platelets, erythrocytes, and other cells that do not  
5 undergo apoptosis, suggest that the nuclear and plasma membrane changes associated with apoptosis are separate "epiphenomena", reflecting independent and unrelated responses to a coordinate rise in  $[Ca^{2+}]$ .

Diaz, et al. (*Blood* 87[7]:2956-2961, 1996) have recently  
10 reported the generation of phenotypically aged phosphatidylserine-expressing erythrocytes by dilauroylphosphatidylcholine (DLPC)-induced vesiculation. Red blood cells were artificially vesiculated with DLPC and assessed for alterations in density, membrane lipid asymmetry  
15 and propensity to be recognized by macrophages *in vitro* and the reticuloendothelial system *in vivo*. The results suggest that vesiculation contributes to alterations in membrane lipid asymmetry and cell characteristics of the aged red blood cell phenotype.

20        Role of cell surface PS in clearance by the RE system.

There is now accumulating data to suggest that cell-surface PS may contribute to the recognition and clearance of senescent, injured, or apoptotic cells by macrophages and other cells of the reticuloendothelial system (J. Savill, et al., *Immunol.*  
25 *Today* 14:131-136, 1993; V. A. Fadok, et al., *J. Immunol.* 148:2207-2216, 1992; J. Connor, et al., *J. Biol. Chem.* 269:2399-2404, 1994). These experiments demonstrate that (i) macrophages have inducible receptors that stereospecifically bind to PS-containing liposomes and to surface-exposed plasma  
30 membrane PS; (ii) selective phagocytosis of apoptotic lymphocytes by stimulated macrophages is observed in the absence of plasma proteins, and this can be inhibited by PS-containing liposomes or by phosphoserine (V. A. Fadok, et al., *supra*, 1992). Consistent with these data, the  
35 circulating lifetime of infused PS-containing liposomes is markedly decreased when compared to liposomes devoid of PS, due to rapid hepato-splenic clearance (T. M. Allen, et al.,

Proc. Natl. Acad. Sci. USA 85:8067-8071, 1988). Similarly, increased exposure of plasma membrane PS during in vitro storage of platelet concentrates may contribute to an accelerated clearance of these cells after transfusion (A. P. Bode, et al., *Thromb. Res.* 39:49-61, 1985; A. P. Bode, et al., *J. Lab. Clin. Med.* 113:94-102, 1989; A. P. Bode, et al., *Blood* 77:887-895, 1991; D. Geldwerth, et al., *J. Clin. Invest.* 92:308-314, 1993; P. Gaffet, et al., *Eur. J. Biochem.* 222:1033-1040, 1994; E. M. Bruckheimer, et al., *J. Leukoc. Biol.* 59, 784-788, 1996; C. Diaz, et al, *supra*, 1996). This possibility is underscored by recent reports documenting increased PS exposure in platelets and red cells during in vitro storage. Evidence that PS exposed on the surface of tumor cells promotes adherence and cytolysis by inflammatory macrophages has also been reported (J. Connor, et al., *Proc. Natl. Acad. Sci. USA* 86:3184-3188, 1989).

Regulation of the transmembrane distribution of PS. It is now well established that phospholipids are normally asymmetrically distributed within the plasma membrane of all blood cells, vascular endothelium, and other cells: the aminophospholipids (including phosphatidylserine (PS) and phosphatidylethanolamine (PE)) reside almost exclusively in the inner membrane leaflet, whereas the outer leaflet is enriched in neutral polar phospholipids, including phosphatidylcholine (PC) and sphingomyelin (B. Roelofsen, *Infection* 19:S206-S209, 1992; A. J. Schroit, et al., *Biochim. Biophys. Acta* 1071:313-329, 1991; P. F. Devaux, *Biochemistry* 30:1163-1173, 1991). It is well-recognized that the transmembrane orientation of plasma membrane PL is central to the regulation of surface-localized enzyme reactions of both complement and coagulation systems and to the recognition and phagocytic clearance of injured, aged or apoptotic cells. It is also now generally accepted that the maintenance of PL asymmetry arises through the activity of a specific transmembrane PL "flippase" with specificity for aminoPL. This aminoPL translocase (APT) has been shown to selectively and vectorially transport PS (> PE), but not neutral PL such



as PC, from outer to inner leaflets of the plasma membrane in a process that is dependent on both  $Mg^{2+}$  and ATP, inhibited by fluoride, o-vanadate or increased  $[Ca^{2+}]_i$ , and inactivated by N-ethylmaleimide (NEM) or pyridyldithioethylamine (PDA) (M. Bitbol, et al., *Biochim. Biophys. Acta* 904:268-282, 1987; M. Seigneuret, et al., *Proc. Natl. Acad. Sci. USA* 81:3751-3755, 1984; J. Connor, et al., *Biochemistry* 26:5099-5105, 1987; P. F. Devaux, et al., *Phys. Lipids* 73:107-120, 1994; A. Zachowski, et al., *Biochemistry* 25:2585-2590, 1986; C. Diaz, et al., *supra*, 1996). In addition to plasma membrane, APT activity has also been identified in the membranes of secretory vesicles and synaptosomes (A. Zachowski, et al., *Nature* 340:75-76, 1989). The  $K_m$  for ATP is approximately 1 mM, and it has been estimated that one molecule of ATP is hydrolyzed for each aminoPL transported (Z. Belezney, et al., *Biochemistry* 32:3146-3152, 1993). Two candidate proteins have been proposed to function as APT: the Rh antigen protein, and a 110-120 kDa  $Mg^{2+}$ -ATPase. Schroit and coworkers (A. J. Schroit, et al., *Biochemistry* 29:10303-10306, 1990) originally proposed that a 32 kDa PS-binding RBC membrane protein that precipitated with antibody to Rh was the erythrocyte APT. Subsequently it was shown that Rh<sub>null</sub> cells deficient in Rh antigen nevertheless exhibit normal APT activity, and very recently, the 32 kDa PS-binding protein that co-precipitates with Rh protein was identified as stomatin, and it was shown that its interaction with PS was not specific for the aminoPL headgroup. This implies that neither stomatin, nor, the Rh protein can provide APT function (J. Desneves, et al., *Biochem. Biophys. Res. Commun.* 224:108-114, 1996). The observed similarity in cation, ATP- and PS-dependence of cellular APT activity to a partially purified  $Mg^{2+}$ -dependent ATPase from RBC led Devaux and associates, and later Daleke, to suggest that APT is a specific  $Mg^{2+}$ -ATPase (A. Zachowski, et al. *supra*, 1989; G. Morrot, et al., *FEBS Lett.* 266:29-32; D. L. Daleke, et al., *Ann. NY Acad. Sci.* 671:468-470, 1992; M. L. Zimmerman, et al., *Biochemistry* 32:12257-12263, 1993). Consistent with this premise, Auland (Auland, et al., *Proc. Natl. Acad. Sci* 91:10938-10942, 1994) demonstrated PS-specific

transport in proteoliposomes reconstituted with an unidentified  $Mg^{2+}$ -ATPase isolated from RBC. Recently, an ATPase II from bovine chromaffine granules has been cloned and sequenced, and evidence has been presented that this enzyme  
5 may exhibit aminoPL translocase activity (X. J. Tang, et al., *Science* 272:1495-1497, 1996).

**Ca<sup>2+</sup> and the collapse of phospholipid asymmetry**

Whereas the rate of spontaneous flip/flop of PL between membrane leaflets is normally quite slow, a substantial rise  
10 in  $[Ca^{2+}]_c$  resulting from agonist-induced activation, programmed cell death, or, secondary to immune injury, initiates rapid transbilayer migration of all plasma membrane PL with net movement of aminoPL to the outer leaflet, collapsing the normal asymmetric distribution (P. Williamson,  
15 et al., *Biochemistry* 31:6355-6360, 1992); F. Bassé, et al., *Biochemistry* 32:2337-2344, 1993; C.-P. Chang, et al., *supra*, 1993; P. Comfurius, et al. *Biochim. Biophys. Acta* 1026:153-160 1990; A. J. Schroit, *supra*, 1991; P. Devaux, *Biochemistry* 30:1163-1173, 1991; J. Connor, et al., *J. Biol. Chem.*  
20 267:19412-19417, 1992). Four different mechanisms have been proposed to account for this induced "scrambling" of plasma membrane PL with net egress of aminoPL to cell surfaces (A. J. Schroit, *supra*, 1991; P. Devaux, *supra*, 1992; P. Devaux, *supra*, 1991; R. F. A. Zwaal, et al., *Biochim. Biophys. Acta*  
25 1180:1-8, 1992): (i) spontaneous collapse of PL asymmetry due to inactivation of vectorial transport by plasma membrane APT; (ii) random scrambling due to transient formation of non-bilayer ( $H_{II}$ -phase) PL domains upon  $Ca^{2+}$ -induced blebbing of plasma membrane vesicles; (iii) direct effects of  $Ca^{2+}$  on  
30 topology and distribution of anionic PLs; (iv) response of a  $Ca^{2+}$ -sensitive protein(s) that facilitates PL transfer between membrane leaflets.

(i) **Spontaneous collapse of PL asymmetry.** APT is inhibited at elevated  $[Ca^{2+}]_c$ , raising the possibility that the concomitant  
35 egress of aminoPL to the cell surface simply reflects spontaneous back-leak ("flop") of the PL distribution that is constitutively maintained by APT (P. Williamson, et al.,

*supra*, 1992; P. F. Devaux, *supra*, 1992; P. Devaux, *supra*, 1991). Nevertheless, inhibition of APT--either by depletion of cellular ATP, or by incubation with fluoride, o-vanadate or NEM--does not in itself cause accelerated transbilayer PL migration or significant cell-surface PS exposure, as long as normally low  $[Ca^{2+}]_c$  is maintained (E. M. Bevers, *et al.*, *supra*, 1991; P. Comfurius, *et al.* *supra*, 1990; B. Verhoven, *et al.*, *Biochim. Biophys. Acta* 1104:15-23, 1992; J. Connor, *et al.* *Biochemistry* 29:37-43, 1990). Conversely, cells genetically deficient in PL scramblase show normal APT activity. Thus it appears that the spontaneous transbilayer migration of plasma membrane PL is inherently very slow at the normal low  $[Ca^{2+}]_c$ , whereas entry of  $Ca^{2+}$  into the cytosol specifically induces rapid movement of PL between plasma membrane leaflets. Whereas the inherent rate of transbilayer migration of PL cannot account for the rapid scrambling observed at elevated  $[Ca^{2+}]_c$ , it is conceivable that interaction of  $[Ca^{2+}]_c$  with APT induces a conformational change that not only inactivates the ATP-dependent inward translocation of aminoPL, but also facilitates selective flop of PS & PE to the outer leaflet. In this context, Bienvenue and associates (Bassé, *et al.*, *supra*, 1993; Gaffet, *et al.*, *Biochemistry* 34:6762-6769, 1995) have reported evidence for transient vectorial egress of PS upon elevation of  $[Ca^{2+}]_c$  in platelet, whereas data from others suggest bidirectional and non-selective transbilayer scrambling of all plasma membrane PL (including PC which is not flipped by APT) at elevated  $[Ca^{2+}]_c$  (P. Williamson, *et al.*, *supra*, 1992; P. Williamson, *et al.*, *Biochemistry* 34:10448-10455, 1995; E. F. Smeets, *et al.*, *Biochim. Biophys. Acta Bio-Membr.* 1195:281-286, 1994; D. Bratton, *J. Biol. Chem.* 269:22517-22523, 1994).

(ii) **Relationship of PS egress to shedding of plasma membrane vesicles.** Data from our laboratory helped establish that surface exposure of PS is intimately related to a process of  $Ca^{2+}$ -induced vesiculation of the plasma membrane, and that formation of such PS-rich plasma membrane "microparticles" contributes to expression of cellular procoagulant activity (C.-P. Chang, *et al.*, *supra*, 1993; P. J. Sims, *et al.*, *supra*,

1989; A. P. Bode, et al., *supra*, 1985; R. F. A. Zwaal, et al.,  
*supra*, 1992; T. Wiedmer, et al., *supra*, 1990; P. J. Sims, et  
al., *J. Biol. Chem.* 263:18205-18212, 1988; K. K. Hamilton, et  
al., *J. Biol. Chem.* 265:3809-3814, 1990; H. Sandberg, et al.,  
5 *Thromb. Res.* 39:63-79, 1985). This correlation between  
microparticle formation and surface exposure of PS suggested  
that the membrane fusion events generating these membrane  
vesicles underlie observed scrambling of plasma-membrane PL,  
presumably through transient formation of  $H_{II}$ -phase PL (C. P.  
10 Chang, et al., *supra*, 1993). Alternatively, prior egress of  
PS to the outer leaflet might create a mass imbalance that  
itself drives plasma membrane evagination and vesiculation  
(P.F. Devaux, *supra*, 1991). In this context, we and others  
have observed that PS migration to the cell surface can  
15 precede membrane vesiculation, and can occur without  
microparticle formation (G. E. Gilbert, et al., *J. Biol. Chem.*  
266:17261-18269, 1991; F. Basse, et al., *Biochemistry*  
32:2337-2344, 1993; P. Gaffet, et al., *supra*, 1995)). Our data  
suggested the participation of a calmodulin-dependent kinase  
20 in  $Ca^{2+}$ -induced vesiculation (T. Wiedmer, et al., *Blood*  
78:2880-2886, 1991). Involvement of a protein kinase in the  
cytoskeletal reorganization required for platelet  
microparticle formation has recently been confirmed (Y. Yano,  
et al. *Biochem. J.* 298:303-308, 1994). This suggests that  
25 whereas membrane fusion may contribute, it neither initiates  
nor is required for PL scramblase function.

(iii) Interaction of  $Ca^{2+}$  with anionic plasma membrane PL.

Ion pairing of  $Ca^{2+}$  with the anionic PS headgroup might  
accelerate transbilayer migration by lowering the energy  
30 barrier to crossing through the hydrophobic membrane interior.  
However,  $Ca^{2+}$  does not directly induce transbilayer migration  
of PL in PS-containing membranes, except when mole% of PS is  
sufficient to induce an  $H_{II}$ -phase and vesicle-vesicle fusion  
(A.L. Bailey, et al., *Biochemistry* 33:12573-12580, 1994; B. de  
35 Kruijff, et al., *Trends Biochem. Sci.* 5:79-81, 1980). Thus  
ion pairing of  $Ca^{2+}$  with inner leaflet PS would not appear to  
directly induce PL flip/flop between membrane leaflets.  
Alternatively, Devaux and associates (J.-C. Sulpice, et al.,

- J. Biol. Chem.* 269:6347-6354, 1994; J.-C. Sulpice, et al., *Biochemistry* 35:13345-13352, 1996) proposed that  $\text{Ca}^{2+}$  binding to phosphatidylinositol 4,5-bisphosphate (PIP2) induces transbilayer migration of other PL, based on the observation
- 5 that adding PIP2 to RBC promoted  $\text{Ca}^{2+}$ -dependent transbilayer movement of PS. Nevertheless, subsequent studies revealed that this incorporation of exogenous PIP2 also induces enough membrane lysis to account for any apparent increase in transbilayer migration of PS (Beyers, et al. *Blood*
- 10 86:1983-1991, 1995).
- (iv) Role of  $\text{Ca}^{2+}$ -sensitive protein(s) in PL redistribution.**
- Elevation of  $[\text{Ca}^{2+}]_i$  is known to give rise to marked changes in several cytoskeletal and membrane proteins that might affect the rate of movement of PL between plasma membrane leaflets.
- 15 For example, several cytoskeletal proteins (including, spectrin and erythrocyte band 4.1) (A. M. Cohen, et al., *Blood* 68:920-926, 1986; S. B. Sato, et al., *Eur. J. Biochem.* 130:19-25, 1983; A. C. Rybicki, et al., *J. Clin. Invest.* 81:255-260, 1988; K. A. Shiffer, et al., *Biochim. Biophys.*
- 20 *Acta* 937:269-280, 1988) have been shown to bind specifically to the PS headgroup, and this interaction potentially serves to trap PS in the inner leaflet of the plasma membrane (P. Comfurius, et al., *Biochim. Biophys. Acta* 983:212-216, 1989). Breaking such interactions, as might occur through interaction
- 25 of  $\text{Ca}^{2+}$  with the PS headgroup, or through proteolytic degradation of cytoskeletal proteins, would potentially dissociate PS from these endofacial contacts and thereby accelerate PS flop to the outer leaflet (P. F. Franck, et al., *supra*, 1985). In this context, it has been reported that
- 30 polyamines inhibit the endogenous PL scramblase activity of the RBC membrane, suggesting that these polycations inhibit interaction of  $\text{Ca}^{2+}$  at its endofacial membrane site (D. L. Bratton, et al., *supra*, 1994; J.-C. Sulpice, et al. *supra*, 1996). Calpain-mediated proteolysis of components of the
- 35 submembrane cytoskeleton can be temporally-correlated to membrane vesiculation and to surface exposure of PS. Nevertheless, inhibition of calpain does not prevent PS egress, and,  $\text{Ca}^{2+}$ -dependent PL scramblase activity is readily

demonstrated in resealed RBC ghosts and inside-out RBC membrane vesicles (IOV) washed free of calpains and other soluble proteases (P. Comfurius, et al., *supra*, 1990; T. Wiedmer, et al., *Biochemistry* 29:623-632, 1990; Fox, et al., *J. Biol. Chem.* 266:13289-13295, 1991; J. E. B. Fox, et al., *J. Cell Biol.* 111:483-493, 1990; P. F. J. Verhallen, et al., *Biochim. Biophys. Acta* 903:206-217, 1987; L. Dachary-Prigent, et al., *Blood* 81:2554-2565, 1993) On the other hand, the possibility that a membrane protein with inherent PL  
10 scramblase activity directly mediates  $\text{Ca}^{2+}$ -induced transbilayer migration of PL was suggested by the observation that this activity is inhibited by sulfhydryl oxidation of membrane proteins with PDA (P. Williamson, et al., *supra*, 1995). Consistent with this finding, we recently reported the  
15 purification and preliminary characterization of an integral RBC membrane protein that, when reconstituted in liposomes, mediates a  $\text{Ca}^{2+}$ -dependent transbilayer movement of PL mimicking plasma membrane PL reorganization evoked upon elevation of  $[\text{Ca}^{2+}]_i$  (F. Bassé, et al., *J. Biol. Chem.*  
20 271:17205-17210, 1996) Evidence that a protein of similar function must also be present in platelets was recently reported by Zwaal (P. Comfurius, et al., *Biochemistry* 35:7631-7634, 1996).

**The Scott syndrome.** Scott syndrome is a bleeding  
25 disorder described in a single patient that reflects impaired expression by activated platelets of the membrane sites that are required for normal assembly of the prothrombinase and tenase enzyme complexes (H. J. Weiss, et al., *Am. J. Med.* 67:206-213, 1979; H. J. Weiss, *Semin. Hematol.* 31:1-8, 1994).  
30 Platelets obtained from this patient secrete and aggregate normally when exposed to various agonists (ADP, thrombin, collagen, complement proteins C5b-9, or calcium ionophore), but when fully activated, exhibit a marked deficiency of membrane binding sites for factors Va and VIIIa, reflecting a  
35 concomitant reduction in the amount of surface-exposed PS (J. P. Miletich, et al., *supra*, 1979; J. Rosing, et al., *supra*,

1985; J. P. Sims, et al., supra, 1989; S. S. Ahmad, et al., supra, 1989).

Although Scott syndrome was originally described as an isolated platelet disorder, it is now clear that other blood cells from this patient, including erythrocytes and lymphocytes, are affected as well (E. M. Bevers, et al. *Blood* 79:380-388, 1992). Measurements that have been made by ourselves and others suggest that Scott platelets and erythrocytes contain normal amounts of PS and other phospholipids, and also exhibit normal aminophospholipid translocase activity (H. J. Weiss, et al., 1979). These cells are defective, however, in their capacity to mobilize PS from inner to outer membrane leaflets in response to elevated  $[Ca^{2+}]_i$ , a response that is now thought to be required for normal prothrombinase and tenase assembly. A search for the molecular defect responsible for the Scott syndrome has to date failed to reveal abnormality in platelet or red cell proteins (probed by 2-dimensional electrophoresis); the agonist-induced elevation of  $[Ca^{2+}]_i$  in Scott platelets is normal; and the calpain and transglutaminase activities of both Scott platelets and RBCs are indistinguishable from normal controls, as assessed by the  $Ca^{2+}$ -induced cleavage or cross-linking of cytoskeletal proteins (P. Comfurius, et al., *Biochim. Biophys. Acta* 815:143-148, 1985).

We have established in in vitro culture EBV-transformed lymphoblast cell lines from this patient and have demonstrated that these immortalized cells exhibit the same phenotype of impaired  $Ca^{2+}$ -induced plasma membrane phospholipid scrambling that is characteristic of the platelets and erythrocytes in this disorder. Our data also establish that this defect propagates through multiple cell divisions and can be corrected by heterokaryon fusion with wild-type cells that exhibit normal plasma membrane phospholipid scrambling (H. Kojima, et al. *J. Clin. Invest.* 94:2237-2244, 1994).

Similar data have recently been reported for a second patient with Scott syndrome, and evidence for a familial inheritance of the cellular defect provided (F. Toti, et al., supra, 1996)). This implies that the molecular basis for this

clinical disorder relates to a gene defect that results in impaired activity of a cellular protein that is expressed in a variety of cell lineages, and that this protein normally mediates the intracellular  $\text{Ca}^{2+}$ -dependent transbilayer movement (or "scrambling") of plasma membrane phospholipids that occurs in response to cell activation, apoptosis, or cell injury. We identify this protein as "phospholipid scramblase", "PL scramblase", and "P37". We mean for "P37" to be synonymous with "phospholipid scramblase or PL scramblase" and refer to these names interchangeably throughout the text.

The loss of normal PL scramblase function in patients with Scott syndrome may relate to either the abnormal expression of an inhibitor of the activity of PL scramblase, a deletion or loss of function mutation in the gene encoding P37 protein, or, a mutation affecting a cofactor of P37 that is required for normal expression of its PL scramblase activity.

Patients with the Scott syndrome defect display abnormal bleeding and a prolongation of the time required for blood clotting (H. J. Weiss, *Semin. Hematol.* 31:1-8, 1994). This implies that activation of PL scramblase is normally required for effective clot formation and for efficient hemostasis, whereas loss or inhibition of PL scramblase activity leads to retarded blood clotting. We therefore propose that the selective activation of PL scramblase function is of potential therapeutic value in the acceleration of hemostasis and in preventing blood loss, whereas the selective inhibition of PL scramblase function is of potential therapeutic value in certain thrombotic disorders characterized by excessive or inappropriate clot formation due to expression of plasma membrane procoagulant activity.

In this application, we identify the cellular component that functions to mediate the  $\text{Ca}^{2+}$ -dependent reorganization of plasma membrane phospholipids and describe methods for preventing egress of PS to the surface of activated, injured, or apoptotic cells.



## BRIEF SUMMARY OF THE INVENTION

The present invention relates to the creation and use of antithrombotic and thrombostatic reagents that depend on the properties of a protein preparation that mediates  $\text{Ca}^{2+}$  dependent transbilayer movement of membrane phospholipids.

The present invention is a preparation of a protein, wherein the protein is a phospholipid scramblase and wherein the protein is approximately 35-37 kD as measured on a 12.5% SDS-polyacrylamide gel under reducing conditions. Preferably, the scramblase comprises residues 59 - 90 of SEQ ID NO:1. More preferably, the preparation comprises residues 1 - 188 of SEQ ID NO:1.

In another embodiment of the present invention, the PL scramblase comprises residues 59 - 90 of SEQ ID NO:1 with conservative or functionally equivalent substitutions.

In the most preferred embodiment of the present invention, the PL scramblase comprises residues 75-392 of SEQ ID NO:4 with conservative or functionally equivalent substitutions.

The present invention is also a DNA sequence encoding the PL scramblase. Preferably, this DNA sequence comprises the nucleic acids 223-1176 of SEQ ID NO:3.

The present invention is also an inhibitor of the PL scramblase activity of P37. This inhibitor may be an antisense nucleotide derived from the DNA sequence of P37. In another embodiment, the inhibitor is a peptide sequence that is a competitive inhibitor of P37 PL scramblase activity. Preferably, the inhibitor is a peptide that either binds to plasma membrane phosphatidylserine, prevents binding of  $\text{Ca}^{2+}$  to P37, or prevents its phosphorylation by cellular protein kinases. In another embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against P37.

The present invention is also a method for preventing the surface exposure of plasma membrane phosphatidylserine, phosphatidylethanolamine and cardiolipin on the surface of in vitro stored platelets or red blood cells. This method comprises the steps of adding an inhibitor of P37 PL

scramblase activity to the stored platelets or red blood cells.

The present invention is also a method for prolonging survival of transplanted organs comprising the step of adding  
5 an inhibitor of P37 PL scramblase activity to an organ perfusate during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically engineering the cells to be transplanted so as to alter their expression of  
10 plasma membrane P37 in order to reduce exposure of PS and other thrombogenic phospholipids at the plasma membrane surface, thereby reducing the risk of infarction due to fibrin clot formation.

The present invention is also a method for prolonging the  
15 *in vivo* survival of circulating blood cells (erythrocyte, platelets, lymphocyte, PMN's, and monocytes) comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the surface of the cells by exposing the blood cells to an inhibitor of P37 PL scramblase activity.

20 The present invention is also a method for preventing the procoagulant activities of erythrocytes in sickle cell disease comprising the step of inhibiting erythrocyte P37 in a sickle cell patient.

The present invention is also a method for treating  
25 autoimmune and inflammatory diseases comprising the step of treating a patient with an inhibitor of the PL scramblase activity of P37.

The present invention is also a method for diagnosing individuals with reduced or elevated capacity for  
30 platelet-promoted or erythrocyte-promoted fibrin clot activity comprising the step of quantitating the cellular expression of P37. This quantitation may take the form of immunoblotting using an antibody to P37, an ELISA assay using an antibody to P37, flow cytometric analysis of the binding of monoclonal  
35 antibody reactive against the predicted extracellular domain of P37 (residues 386 - 392 of sequence disclosed in SEQ ID NO:4) or using oligonucleotides derived from P37 cDNA and the polymerase chain reaction. In one method of the present

invention, the quantitation is performed by isolating P37 from a patient blood sample, measuring the amount of P37 isolated and comparing the measurement with a control sample. The measurement may be by isolating P37 from a patient blood sample and measuring via densitometry the amount of P37 protein electrophoresed in a stained electrophoretic gel.

It is an object of the present invention to provide a preparation of a PL scramblase.

It is another object of the present invention to provide an inhibitor of P37 PL scramblase activity.

It is another object of the present invention to provide an antithrombotic agent.

It is another object of the present invention to create cells, tissue, and organs for transplantation that have increased potential for survival and reduced potential for causing fibrin clot formation and vascular thrombosis when grafted into a recipient host.

Other objects, advantages and features of the present invention will become apparent after one of skill in the art reviews the specification, claims and drawing herein.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1A is a bar graph depicting the amount of PL scramblase activity as a function of chromatography column fraction number. Fig. 1B represents a 12.5% SDS-PAGE in which the fractions described in Fig. 1A have been electrophoresed. The actual 37 kD bands are depicted by dark rectangles.

Fig. 2 is a diagram of the bidirectional movement of NBD-PS across lipid bilayers reconstituted with P37.

Fig. 3 is a graph describing the kinetics of PS translocation by reconstituted P37.

Fig. 4 is a graph describing calcium dependence of P37 activity in proteoliposomes and in erythrocyte IOVs.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is the purification and characterization of an approximately 35-37 kD membrane protein from human erythrocytes that promotes a  $\text{Ca}^{2+}$ -dependent transbilayer redistribution of membrane phospholipids including PS and PC, with properties similar to the PL scramblase activity that is evoked upon elevation of  $\text{Ca}^{2+}$  in the cytosol of erythrocytes and other cells. We have named this membrane protein "P37." We mean for "P37" to be synonymous with "phospholipid scramblase or PL scramblase" and refer to these names interchangeably throughout the text. By "phospholipid scramblase or PL scramblase activity," we mean the  $\text{Ca}^{2+}$  dependent transbilayer movement of plasma membrane phospholipid.

In one embodiment, the present invention is a protein preparation of P37. Preferably, the protein comprises residues 59 - 90 of SEQ ID NO:1. More preferably, the PL scramblase comprises residues 1 - 188 of SEQ ID NO:1. Most preferably, the protein comprises residues 75 - 392 of SEQ ID NO:4.

In another embodiment, the protein comprises conservative substitutions or functionally equivalent residues of the residues described in the paragraph above. By "functionally equivalent" we mean that the equivalent residues do not inhibit or disrupt the activity of the PL scramblase preparation.

The examples below demonstrate one method of isolating P37 from human erythrocytes. After examination of the specification below, other methods of protein isolation will become apparent to one of skill in the art. The examples below also describe an assay for the measurement of PL scramblase activity. A suitable preparation of the present invention would have a PL scramblase activity of at least 10% that of the preparation described below in the examples. Preferably, the activity would be at least 50% that of the examples described below.

The present invention is also a recombinant DNA sequence encoding P37. A preferable DNA sequence encoding P37 would

comprise the nucleic acids of SEQ ID NO:2. A more preferable DNA sequence encoding P37 would comprise the nucleic acids 223 - 1176 of SEQ ID NO:3. One of skill in the art of molecular biology would know how to obtain DNA sequences encoding the PL  
5 scramblase. For example, one might sequence P37 directly via standard protein sequencing techniques as we report below in Example C. The peptide sequence could be analyzed to provide oligonucleotide probes for a human cDNA leukocyte library. (One such cDNA library is available from Invitrogen in a  
10 pCDNA3 vector.)

Residues 59 - 90 of SEQ ID NO:1 are the amino acid sequence that we obtained of a cyanogen bromide fragment of the PL scramblase. SEQ ID NO:1 is the translated product of ATCC clone 962235 and is a longer amino acid sequence  
15 containing residues 59 - 90. Residues 75 - 392 of SEQ ID NO:4 is the predicted full-length sequence of the P37 polypeptide, based on analysis of the open reading frame of SEQ ID NO:3.

By use of probes obtained from other domains of the PL scramblase one would then be able to isolate a cDNA clone  
20 encoding the entire P37 protein sequence. Sequence identified as SEQ ID NO:3 contains the entire open reading frame encoding P37 as well as flanking residues of 5' and 3' untranslated sequence. The full-length translation of SEQ ID NO:3 is identified as SEQ ID NO:4. (The symbol Xaa indicates a stop.)  
25 Analysis of the open reading frame of this sequence identifies residues 75 - 392 of SEQ ID NO:4 as comprising the full-length protein sequence of P37. No signal peptide sequence is identified between residues 75 - 392 of SEQ ID NO:4, indicating that this is also likely to be the length of the mature  
30 polypeptide after post-translational processing. The calculated molecular weight of the polypeptide containing residues 75-392 of SEQ ID NO:4 is 35,049 daltons and closely approximates the molecular weight of P37 of 35-37 kDa as estimated by apparent mobility in SDS-PAGE.

35 Most preferably, the preparation would have at least 90% the activity of the PL scramblase preparation described below.

In one embodiment of the invention, the PL scramblase is isolated from erythrocyte membranes. In another embodiment,

the protein is produced by bacteria cells, such as *E. coli* cells, insect cells, or yeast, preferably *in vitro* cultures that are transfected with plasmid or viral vectors containing cDNA sequences identified at SEQ ID NO:2 in the correct  
5 reading frame (3) or cDNA sequences identified at SEQ ID NO:3 in correct reading frame (1). The vector can be chosen from among protein expression vectors known to those skilled in the art. Preferable viral vectors include retrovirus, adenovirus, and baculovirus vectors.

10 The present invention is also an inhibitor of the PL scramblase activity of P37. In one embodiment, the inhibitor is an antisense nucleotide derived from the DNA sequence encoding P37. One of skill in the art would know how to create such an antisense nucleotide from the cDNA sequence of  
15 P37. In another embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against P37. One of skill in the art would know how to make an antibody preparation from the purified protein preparation described below. For example, one could isolate the purified P37  
20 protein from the electrophoretic gel band and use this preparation to inject into an antibody-producing host, such as a rabbit, sheep, goat, rat, or mouse. In addition to polyclonal immune serum, the antibody-producing splenic lymphocytes from such animals can be obtained to prepare  
25 monoclonal antibody reactive with specific peptide segments of P37. Such methods are well-known to those skilled in the art and include myeloma fusion and the *in vitro* culture, cloning and selection of the resulting hybridoma cells to obtain monoclonal IgG. In a preferred embodiment, recombinant  
30 antibody fragments consisting of antigen-binding Fv or ligated single chain Ig ScFv can be prepared from a cDNA library derived from the polyA mRNA of the immune splenic lymphocytes. In this embodiment, PCR is used to selectively amplify cDNA encoding the CDR3 hypervariable domains of the heavy and light  
35 Ig chains, this cDNA is then ligated into appropriate phage expression vectors, and the recombined or fused single chain Fv are then expressed as soluble protein and on the surface of the phage particles that are propagated in *E. Coli* or other

suitable host cell. The resulting express recombined monoclonal Ig Fv on their surface and can be panned and cloned to isolate unique cDNA encoding for specific monoclonal reagents that bind to select epitopes on P37 antigen. This  
5 unique cDNA can then amplified and used to express large quantities of the desired monoclonal antibody reagent in an appropriate expression system. In another embodiment, the anti-P37 Fv reagent is humanized to reduce its antigenicity for purposes of injection into a human host. The humanized Fv  
10 is prepared by converting the nucleotide segments of the murine-derived cDNA to the homologous human sequence in those segments of the heavy and light chain Ig polypeptides that are flanking on either side of the actual antigen combining site. The methods for preparation of these various recombinant  
15 immunoglobulin reagents are well-known to those skilled in the art and are also commercially available in kit form (e.g. Pharmacia ScFv).

In another embodiment, the inhibitor is a peptide derived from P37. This peptide sequence is an antagonist or  
20 competitive inhibitor of P37 PL scramblase activity. The inhibitor activity could take the form of competing with the binding of native P37 with plasma membrane phosphatidylserine or competing with native P37 in calcium binding. One of skill in the art would realize that one could examine different  
25 peptide fragments of P37 for suitability as a competitive inhibitor.

The present invention is also a method for preventing the surface exposure of plasma membrane phospholipids, such as phosphatidylserine, phosphatidylethanolamine and cardiolipin,  
30 on the surface of *in vitro* stored platelets or red blood cells by adding an inhibitor of the PL scramblase activity of P37 to the stored platelets or red blood cells.

The present invention is also a method for prolonging survival of transplanted organs and grafts comprising the step  
35 of adding an inhibitor of P37 PL scramblase activity to an organ perfusate during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically

engineering the cells to be transplanted so as to alter their expression of plasma membrane P37 in order to reduce exposure of PS and other thrombogenic phospholipids at the plasma membrane surface, thereby reducing the risk of infarction due to fibrin clot formation.

Therefore, in one embodiment, the present invention is a genetically engineered cell for transplantation into a human or animal wherein the cell has a lowered PL scramblase expression. Preferably, the cell expresses no PL scramblase. Preferably, this cell comprises a nucleotide molecule which is expressed by the cell and which codes for protein inhibiting the activity of PL scramblase. In another preferable embodiment, the promotor of the PL scramblase gene is altered to either increase or decrease the expression of the gene. One of skill in the art of molecular biology would envision methods to create these altered cells.

Preferably, the engineered cell is selected from the group consisting of endothelial cells, fibroblasts, epithelial cells, skeletal cells, cardiac and smooth muscle cells, hepatocytes, pancreatic islet cells, bone marrow cells, astrocytes, and Schwann cells. The present invention is also a prosthesis for implantation in an animal or human having the genetically engineered cells attached thereto. In one embodiment, the prosthesis is a vascular graft.

The present invention is also a method for prolonging the *in vivo* survival of circulating blood cells comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the circulating blood cells plasma membrane P37. One may also wish to prevent the procoagulant properties of erythrocytes in sickle cell disease by inhibiting erythrocyte P37 in a sickle cell patient.

The present invention is also a method for treating autoimmune and inflammatory diseases, such as disseminated intravascular coagulation, vascular thrombosis, fibrin generation during cardiopulmonary bypass procedures, rheumatoid arthritis, systemic lupus erythematosus, thrombotic thrombocytopenic purpura, heparin-associated thrombosis, and



organ transplant rejection comprising the step of treating a patient with an inhibitor of the PL scramblase activity P37.

The present invention is also a method for diagnosing individuals with reduced or elevated capacity for  
5 platelet-promoted or erythrocyte-promoted fibrin clot activity by quantitating the level of cellular expression of P37 in the individual. This method may be performed by using an antibody to P37 in an immunoblot or ELISA method. The method may also be performed using oligonucleotides derived by P37 cDNA in the  
10 polymerase chain reaction. In another embodiment, the method may be performed by isolating P37 from a whole blood sample, measuring the amount of P37 isolated and comparing the measurement with a control sample.

One may wish to use the protein preparation of the  
15 present invention as a hemostatic agent by topically applying the protein preparation to a wound area in a freely bleeding patient.

#### EXAMPLES

##### A. Experimental Procedures.

20 **Abbreviations used.** PL, phospholipid(s); PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine; NBD-PS,  
25 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine; OG, N-Octyl- $\beta$ -D-glucopyranoside; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; APLTase, aminophospholipid translocase; IOVs, inside-out vesicles.

**Materials.** Egg yolk phosphatidylcholine (PC), brain  
30 phosphatidylserine (PS), 1-palmitoyl 2-oleoyl phosphatidic acid, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine (NBD-PC) and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine (NBD-PS) were obtained from Avanti  
35 Polar Lipids. N-Octyl- $\beta$ -D-glucopyranoside (OG) was purchased

from Calbiochem. Sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ , Sigma) was freshly dissolved in 1 M Tris pH 10 at a concentration of 1 M.

**PL Scramblase isolation.** Human erythrocytes were obtained from The Blood Center of Southeastern Wisconsin.

5 Washed erythrocytes were lysed in 10 vol of ice cold 5 mM sodium phosphate, 1 mM EDTA, pH 7.4, washed free of hemoglobin, and solubilized at a final protein concentration of 3.5 mg/ml in the presence of 100 mM OG in buffer A (20 mM Tris, 0.1 mM EGTA, pH 7.4). After incubation for 30 minutes

10 at 4°C with continuous stirring, samples were centrifuged at 100,000 x g for 30 minutes. The cytoskeleton-depleted supernatants were loaded on a Poros 20 HQ anion-exchange column (PerSeptive Biosystems) equilibrated with buffer B (20 mM Tris, 0.1 mM EGTA, 50 mM OG, pH 7.4). Elution was

15 performed with a gradient of 0-400 mM NaCl in buffer B. Active fractions were pooled, subjected to a buffer exchange by PD 10 gel filtration (Pharmacia) into buffer C (50 mM acetate, 0.1 mM EGTA, 50 mM OG, pH 5.0), and loaded on a Poros 20 HS cation-exchange column (PerSeptive Biosystems)

20 equilibrated in buffer C. The breakthrough fraction was passed through a PD 10 column equilibrated in buffer D (25 mM PIPES, 1 mM  $\text{CaCl}_2$ , 50 mM OG, pH 6.0) and loaded on a Reactive Red 120 6% agarose column (Sigma). Elution was performed with a gradient of 0-1.5 M NaCl in buffer D. Active fractions were

25 pooled and further purified on a Bio-Sil SEC-250 size exclusion column (Bio-Rad) equilibrated with buffer D in which  $\text{CaCl}_2$  was replaced by 0.1 mM EGTA. Due to losses encountered through size exclusion chromatography, enriched fractions of PL scramblase were routinely prepared by replacing this step

30 by a second anion exchange fractionation on Mono Q (Pharmacia). All column chromatography procedures were performed at room temperature on a BioCAD 20 perfusion chromatography workstation (PerSeptive Biosystems).

**Reconstitution into proteoliposomes.** A mixture of PC and

35 PS (9:1 molar ratio) was dried under a stream of nitrogen and resuspended in buffer E (100 mM Tris, 100 mM KCl, 0.1 mM EGTA,

pH 7.4) by vigorous vortexing. Protein samples were mixed with liposomes at a final lipid concentration of 4 mg/ml in the presence of 60 mM OG. Detergent was removed by dialysis overnight at 4°C against 200 vol of buffer E containing 1 g/L SM2-Biobeads (BioRad).

**Labeling of proteoliposomes with NBD-PL.** Proteoliposomes were labeled with fluorescent NBD-PC or NBD-PS (0.25 mole%) selectively incorporated into outer, inner, or both membrane leaflets (E. M. Bevers, et al., *supra*, 1995; J. C. McIntyre and R. G. Sleight, *Biochemistry* 30:11819-11827, 1991). Outer leaflet-labeled proteoliposomes were prepared by addition of NBD-PL (in DMSO) to the pre-formed proteoliposome suspension. Symmetrically-labeled proteoliposomes were prepared by adding NBD-PL to the PC-PS mixture in CHCl<sub>3</sub>, before drying lipids under nitrogen (above). Addition of protein sample in OG and dialysis against buffer E was then performed as above. Proteoliposomes with fluorescent NBD-PL located exclusively in the inner leaflet of the bilayer were obtained by incubating symmetrically-labeled proteoliposomes with 20 mM dithionite for 1 minute, in order to chemically and irreversibly quench fluorescent analogs located in the outer leaflet (J. C. McIntyre and R. G. Sleight, *Biochemistry* 30:11819-11827, 1991). Vesicles were gel filtered in buffer E to remove unincorporated material and used immediately for assay of PL scramblase activity.

**PL Scramblase activity.** PL scramblase activity was measured using a modification of an assay previously described (E. M. Bevers, et al., *supra*, 1995; J. C. McIntyre and R. G. Sleight, *supra*, 1991). Routinely, NBD-PL outside-labeled proteoliposomes (0.4 mg/ml PL final concentration) were incubated at 37°C in buffer E in presence or absence of 2 mM CaCl<sub>2</sub>. At times indicated in figure legends, proteoliposomes were diluted 25-fold in buffer E containing 4 mM EGTA, and transferred to a stirred fluorescence cuvet at 23°C. Initial fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter; excitation at 470 nm, emission at 532 nm), and 20 mM

dithionite added with fluorescence continuously monitored for total of 90 seconds. The difference in residual (non-quenchable) fluorescence observed for samples preincubated at 37°C in presence vs. absence of 2 mM CaCl<sub>2</sub> was attributed to Ca<sup>2+</sup>-induced change in NBD-PL located in the outer leaflet, and hence accessible to dithionite (J. C. McIntyre and R. G. Sleight, *supra*, 1991). Maximum (100%) dithionite quenching was determined by addition of 1% Triton X-100. In all cases, NBD fluorescence was corrected for photobleaching (<6%; measured in absence of dithionite). Ionized [Ca<sup>2+</sup>] (Fig. 4) was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

**Trypsination.** Samples containing active PL scramblase were incubated for 3 hours at 37°C in the presence of 2 µg/ml trypsin in 100 mM Tris, 0.1 mM EGTA, pH 8.4, and the reaction was stopped by addition of 1 mM diisopropylfluorophosphate. Samples incubated in absence of trypsin or in presence of trypsin plus 4 µg/ml soybean trypsin inhibitor served as controls. Residual PL scramblase activity of each sample was then determined following reconstitution into NBD-PS outside-labeled liposomes. Residual PL scramblase detected in trypsin-treated samples was normalized to that observed for identically matched samples incubated without trypsin.

**Preparation of erythrocyte IOVs.** Erythrocyte inside out vesicles (IOVs) were prepared essentially by methods described by Steck and Kant (T. L. Steck and J. A. Kant, *Methods Enzymol.* 31a:172-180, 1974) with minor modifications. Assays for acetylcholinesterase (outside) and glyceraldehyde 3-phosphate dehydrogenase accessibility (inside) (T. L. Steck and J. A. Kant, *supra*, 1974) confirmed that >90% of these IOVs were properly oriented and resealed.

**PL Scramblase activity in IOVs.** IOVs were suspended in 10 mM Tris, 140 mM KCl, 7.5 mM NaCl, 0.1 mM EGTA, pH 7.4 at 100 µg protein/ml. NBD-PLs (PC or PS) were added to a final

0.25 mole% of total PL. After incorporation of label, IOVs were incubated in presence 0-2 mM  $\text{CaCl}_2$  for 15 minutes at 37°C. For each sample, percentage of NBD-PL displaced from the external to inward-facing membrane leaflet was determined by the BSA "back-exchange" method as previously described (E. M. Bevers, et al., *supra*, 1995). PL scramblase activity was evaluated by comparing the amount of NBD-PL moved to the inward-facing leaflet of IOVs in the presence of  $\text{Ca}^{2+}$  to that observed in the presence of 0.1 mM EGTA. Transport of dithionite by erythrocyte membrane anion exchanger precludes use of this quencher to monitor NBD-PL distribution in IOVs (T. Pomorski, et al., *Mol. Membr. Biol.* 11:39-44, 1994).

## B. Results and Discussion

**Purification and membrane reconstitution of erythrocyte PL scramblase.** Human erythrocyte membrane proteins depleted of cytoskeleton were solubilized with OG and initially fractionated by anion exchange chromatography. Each eluting fraction was reconstituted into PL vesicles and then 0.25 mole% NBD-PS added to the outer leaflet. After incubation in the presence of either 0 or 2 mM  $\text{Ca}^{2+}$ , the time-dependent distribution of NBD-PS between inner and outer leaflets was determined (see *Experimental Procedures* and below). A  $\text{Ca}^{2+}$ -dependent movement of NBD-PS from outer to inner membrane leaflets was detected in only those vesicles reconstituted with proteins eluting from Poros 20 HQ between 125 mM-200 mM  $\text{NaCl}$ . Such activity was not detected in any other column fraction, including those enriched with the predominant erythrocyte membrane band 3 protein, nor was this activity found in unbound flow-through fractions (not shown). Of note, erythrocyte band 3 protein has been reported to exhibit intrinsic "flipping activity" for anionic PL, based on the capacity of the anion transport inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid to further attenuate the slow spontaneous ( $\text{Ca}^{2+}$ -independent) migration PL across the erythrocyte membrane (A. Vondenhof, et al., *Biochemistry* 33:4517-4520, 1994).

Through subsequent purification, this PL scramblase activity was found to co-purify with a ~37 kDa protein band that we were able to enrich to apparent homogeneity (Fig. 1). Fig. 1 illustrates final stage of purification of erythrocyte PL scramblase activity. Pool of activity eluting from Reactive Red 120 was concentrated and applied to a 600 x 7.5 mm BioSil SEC250 column. Flow rate was 0.2 ml/min and 1.0 ml fractions were collected. 100  $\mu$ l aliquots of each column fraction were reconstituted into liposomes containing NBD-PS exclusively in the outer leaflet and assayed for PL scramblase activity. Samples were also concentrated 10-fold and analyzed by 12.5% SDS-PAGE under reducing conditions, developed with silver staining. Relative PL scramblase activity is plotted directly above the corresponding gel lane. Only data for fractions eluting about peak of activity (fraction #12) are shown. See Experimental Procedures. Fractions 11 and 13 contained additional bands not represented in Fig. 1B.

The activity of this PL scramblase was destroyed by trypsin (Table 1), confirming that activity detected in the reconstituted proteoliposome derived from the purified protein fraction, and not other potentially co-eluting material. In this context, PL scramblase activity has recently been attributed to an interaction of  $\text{Ca}^{2+}$  directly with membrane lipid. As noted above (Introduction), Sulpice, et al. (J. C. Sulpice, et al., *supra*, 1994) concluded that  $\text{Ca}^{2+}$ -binding to  $\text{PIP}_2$  located in the inner leaflet of the erythrocyte membrane induces transbilayer PL scrambling, although this conclusion was not supported by a subsequent study (E. M. Bevers, et al., *supra*, 1995). Alternatively, Bratton proposed that cytoplasmic polyamines contribute to maintenance of PL asymmetry by shielding anionic head groups from  $\text{Ca}^{2+}$ , and that enhanced transbilayer movement of PL might arise through loss of polyamine-membrane associations at increased intracellular  $\text{Ca}^{2+}$  (D. L. Bratton, *supra*, 1994). Although our studies do not address a potential influence of these cytoplasmic components on PL scramblase activity, it should be noted that polyamines would be removed through our lysis and solubilization procedures. Finally, sensitivity of PL

scramblase to inactivation by trypsin (Table 1) excludes the possibility that the activity we observe arises from contaminating  $\text{PIP}_2$  or polyamine. Although data of Fig. 1 and Table 1 suggest that PL scramblase is a low abundance integral membrane polypeptide of ~37 kDa, we cannot now exclude the possibility that observed activity resides in a trace co-purifying membrane protein that goes undetected by silver staining.

**Characteristics of PL transport mediated by isolated PL scramblase.** Proteoliposomes reconstituted with purified PL scramblase remained impermeant to dithionite and stable to spontaneous NBD-PS exchange in absence of  $\text{Ca}^{2+}$  (Fig. 2).

Fig. 2 illustrates bidirectional movement of NBD-PS across lipid bilayers reconstituted with PL scramblase. PL scramblase was reconstituted into 90% PC, 10% PS membranes trace-labeled with 0.25% NBD-PS, initially located either in the inner membrane leaflet (*inside-labeled*), randomly distributed between leaflets (*symmetrically-labeled*), or exclusively in the outer leaflet (*outside-labeled*). Vesicles were incubated 3 hours at 37°C in presence of either 0.1 mM EGTA (*dotted lines*) or 2 mM  $\text{Ca}^{2+}$  (*solid lines*) and reaction stopped with EGTA. Distribution of NBD-PS in outer leaflet was determined by quenching with 20 mM dithionite, time of addition indicated by arrow (see Experimental Procedures). Ordinate denotes NBD fluorescence, normalized to  $t=0$ ; abscissa denotes fluorescence acquisition time (sec). Dashed line indicates level of fluorescence detected after addition of 1% Triton X-100 to each vesicle suspension in presence of dithionite (100% quenching). In this experiment, observed  $\text{Ca}^{2+}$ -dependent transbilayer movement of NBD-PS equaled  $8 \pm 2\%$  (*inside labeled*),  $0 \pm 1\%$  (*symmetrically-labeled*), and  $8 \pm 1\%$  (*outside-labeled*). Fig. 2 illustrates the data of single experiment, representative of at least three separate experiments so performed.

As also illustrated by Fig. 2,  $\text{Ca}^{2+}$ -induced PL scramblase activity was observed to be bidirectional, induced sequestration of NBD-PS initially distributed only in the

outer membrane leaflet equaling induced exposure of NBD-PS initially distributed only in the inner leaflet. In proteoliposomes prepared with NBD-PS randomly distributed between membrane leaflets, no movement of probe was detected upon  $\text{Ca}^{2+}$  addition, consistent with one-for-one PL exchange between membrane leaflets. Similar results were obtained when NBD-PC substituted for NBD-PS, and movement of either NBD-labeled PL was unaffected by replacement of matrix PS by phosphatidic acid (data not shown). These results imply that the PL scramblase activity exhibited by lipid vesicles reconstituted with this 37 kDa protein is non-selective for PS versus PC, and facilitates both inward and outward movement of PL between membrane leaflets. Assuming that movement of PL initiated by addition of  $\text{Ca}^{2+}$  arises through activation of only those proteins oriented to expose  $\text{Ca}^{2+}$ -binding site(s) at the external face of the liposome, these data imply that each PL scramblase mediates bidirectional and non-selective one-for-one exchange of PL between membrane leaflets. Although it has been suggested that elevation of cytosolic  $\text{Ca}^{2+}$  initiates a vectorial movement of PS and PE from inner to outer plasma membrane leaflets (Bassé, et al., *supra*, 1993; P. Gaffet, et al., *supra*, 1995), more recent data suggest that this apparent egress of inner leaflet PS arises through random bidirectional exchange of PL residing in both leaflets of the plasma membrane (P. Williamson, et al., *supra*, 1992; E. F. Smeets, et al., *supra*, 1994; P. Williamson, et al., *supra*, 1995), consistent with the activity we observe in PL scramblase reconstituted proteoliposomes. The fact that these proteoliposomes remain impermeant to dithionite (see Fig. 2), excludes the possibility that this apparently random and bidirectional movement of lipid arises through lytic disruption of lipid bilayer. Nevertheless, we cannot exclude the possibility that an initially vectorial transport of PL in these small vesicles is masked by a counterbalancing exchange to maintain mass balance between membrane leaflets, as might be imposed by head group packing constraints.

The mechanism by which  $\text{Ca}^{2+}$  interaction with this protein initiates transbilayer movement of PL remains unresolved. To



be noted, we observed only a requirement for  $\text{Ca}^{2+}$  with no requirement for either  $\text{Mg}^{2+}$  or ATP. In presence of 2 mM  $\text{Ca}^{2+}$ , lipid movement catalyzed by PL scramblase obeyed pseudo-first order kinetics with initial rates that increased with  
5 concentration of protein, consistent with a facilitated transport or carrier mechanism (Fig. 3).

Fig. 3 diagrams the kinetics of PS translocation by reconstituted PL scramblase. Proteoliposomes were prepared with a constant amount of PL and 2-fold dilutions of purified  
10 PL scramblase (*Experimental Procedures*). NBD-PS (0.25 mole%) was added to outer leaflet and labeled proteoliposomes incubated at 37°C in presence of 2 mM  $\text{Ca}^{2+}$ . At times indicated (*abscissa*) samples were analyzed for the amount of NBD-PS that migrated from outer to inner leaflets, according  
15 to assay shown in Fig. 2. All data are corrected for spontaneous transbilayer migration of NBD-PS ( $\leq 1\% \text{ hr}^{-1}$ ) measured for identical samples incubated with 0.1 mM EGTA, substituting for  $\text{Ca}^{2+}$ . Fig. 3 illustrates the data at highest protein concentration (○); 2-fold (■); 4-fold (●) and 8-fold  
20 dilutions (□) are indicated. Experimental points at each protein concentration were fitted to first-order exponential rate equation (Sigma Plot; dotted lines). *Insert*: Derived initial rates ( $V_i$ ; *ordinate*) plotted against normalized protein concentration (*abscissa*). Data of single experiment,  
25 representative of two so performed.

Derived rate constants for transbilayer lipid movement (at 37°C) ranged between 0.45 - 0.74  $\text{hr}^{-1}$  over an eight-fold range of protein/lipid ratio. Whereas this activity deviated from anticipated linearity with increased added protein per  
30 lipid (*insert*, Fig. 3), this may reflect an inherent limitation in efficiency of protein reconstitution into the liposome membrane (*below*). The apparent rate of  $\text{Ca}^{2+}$ -evoked transbilayer movement attained in the reconstituted system ( $t_x$  -2 hr; Fig. 3) is approximately one-fourth of the observed  
35 rate of PL scrambling ( $t_x$  -0.5 hr; P. Williamson, et al., *supra*, 1992) induced in  $\text{Ca}^{2+}$ -ionophore treated erythrocytes, suggesting somewhat reduced activity in the reconstituted system. Whether this reduced activity reflects partial

denaturation of PL scramblase, or, our inability to incorporate amounts of this protein equivalent to that present in the erythrocyte membrane, remains to be determined.

**Calcium dependence.** In order to confirm that the activity of reconstituted PL scramblase mimicked intrinsic PL scramblase activity of the erythrocyte membrane, we compared  $[Ca^{2+}]$ -dependence of PL movement in proteoliposomes reconstituted with purified protein to that observed in erythrocyte IOVs (Fig. 4). Fig. 4 diagrams  $Ca^{2+}$  dependence of PL scramblase activity in proteoliposomes & erythrocyte IOVs. PL scramblase activity of reconstituted proteoliposomes (closed symbols) and erythrocyte IOVs (open symbols) was determined as described in *Experimental Procedures*, and plotted as function of external free  $[Ca^{2+}]$  ( $\bullet, \circ$ ). Data for each sample were normalized to maximum PL scramblase activity, measured at 2 mM  $Ca^{2+}$ . Squares ( $\blacksquare, \square$ ) denote PL scramblase activity detected when  $Mg^{2+}$  substituted for  $Ca^{2+}$ . Data of single experiment, representative of three so performed.

In both IOVs and isolated protein reconstituted liposomes, PL scramblase activity was found to obey a sigmoidal dose-response to  $Ca^{2+}$ , with saturation at  $\sim 100 \mu M$   $[Ca^{2+}]$ , and half-maximal activities attained between 20-60  $\mu M$   $Ca^{2+}$ . In both cases, little effect of  $Mg^{2+}$  was observed. These results are similar to data previously reported for  $Ca^{2+}$ -induced movement of plasma membrane PL in ionophore-treated erythrocytes, resealed erythrocyte ghosts, and other cells (H. Kojima, et al., *Biochemistry* 33:4517-4520, 1994; B. Verhoven, et al., *Biochim. Biophys. Acta* 1104:15-23, 1992).

PL scramblase activity found in erythrocyte membranes is also observed in platelets, lymphocytes and other cells and is thought to play a central role in initiation of fibrin clot formation and in recognition of apoptotic and injured cells by the reticuloendothelial system. It is not known whether the same protein is responsible for PL scramblase activity found in other cells, or whether such proteins are cell-specific. A protein common to all blood cells is suggested by the

observation that the genetic defect in PL scramblase activity arising in Scott syndrome affects all hematologic lineages. Further insight into the cellular distribution of the 37 kDa protein that we have now isolated from normal erythrocytes awaits purification of sufficient protein for sequencing, and to develop specific antibodies.

Table 1. Inactivation of PL scramblase by trypsin

	Treatment		PL scramblase activity* (% of control)
	Trypsin	Soybean Trypsin Inhibitor	
10	-	-	(100)
	-	+	100
	+	-	13
	+	+	100

\*PL scramblase in response to 2 mM  $\text{Ca}^{2+}$ , expressed as percent of activity of untreated control. Incubation with trypsin and reconstitution of digested protein into liposomes for PL scramblase assay are detailed under *Experimental Procedures*. Range of measured activities  $\pm 5\%$ . Representative of two separate experiments.

C. Amino Acid and Nucleotide Sequence of the PL Scramblase. We purified the PL scramblase by the methods described above. The resulting protein was subjected to further purification by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein band visualized by staining with Coomassie brilliant blue, and a slice of the wet gel containing the P37 protein band migrating between ~35-37 kDa excised. This gel slice was sent to the Protein and Carbohydrate Structure Facility at University of Michigan, Ann Arbor, Michigan for cyanogen bromide digestion and internal peptide sequencing. Peptide sequence was obtained from a 2 kD cyanogen bromide fragment derived from the protein contained in the gel slice. The amino acid sequence we obtained is disclosed in residues 59 - 90 of SEQ ID NO:1.

We obtained an exact match for these residues with a translation product of a cDNA reported in Genbank at AA14325.

This sequence includes the predicted methionine (residue 58 of SEQ ID NO:1) located 5' to the preferred locus of cyanogen bromide peptide bond cleavage, yielding a total of 33 residues of continuous identity between the deduced partial internal  
5 sequence from P37 and the translation product of the cDNA clone reported in Genbank at AA143025. The entire nucleotide sequence of this cDNA clone (ATCC 962235) is reported at SEQ ID NO:2. Sequence reported at SEQ ID NO:1 is the entire  
10 translated sequence of SEQ ID NO:2, using the correct translation reading frame that we deduced from comparison of the cDNA clone with residues 58 - 90 of SEQ ID NO:1.

Using methods standard to those skilled in the art, we probed human leukocyte, B-lymphocyte, fetal adrenal, and K562 (a human erythro-leukemic cell line) cDNA libraries by PCR,  
15 using oligonucleotide primers designed to anneal specifically to SEQ ID NO:2. The PCR reactions revealed that cDNA overlapping SEQ ID NO:2 of the expected number of residues was contained in each of these cDNA libraries. This result indicates that mRNA encoding sequence contained in P37 protein  
20 purified from human erythrocyte membrane is also expressed in multiple human cell lines of both erythroid and non-erythroid origin. Based on evidence for the relative abundance of cDNA overlapping SEQ ID NO:2 in the Clontech lambda gt11 K562 library, we selected this library to amplify additional cDNA  
25 sequence flanking SEQ ID NO:2.

— Using methods standard to those skilled in the art, we PCR amplified the flanking DNA sequences 5' and 3' to sequence ID NO:2 from the K562 lambda gt11 cDNA library (Clontech, Inc) with oligonucleotide primers designed to anneal to sequence ID  
30 NO:2 in combination with oligonucleotide primers designed to anneal to regions of the Clontech lambda gt11 vector in the vicinity of the multiple cloning site of the vector. The resulting PCR-amplified cDNA was purified and sequenced. From these PCR-amplified 5' and 3' flanking sequences, partial  
35 overlapping identities were found in cDNA reported in Genbank at AA143025, AA056199, AA054476, AA151006, AA171663, D61890, H42131, R76171, R66275, R82537, W90481 and these multiple sequences used in combination with direct sequence obtained

from our PCR products to deduce and to confirm the sequence reported at SEQ ID NO:3.

SEQ ID NO:4 is the entire translated sequence of SEQ ID NO:3, using the correct translation reading frame that we deduced from comparison of the cDNA clone with peptide residues 58 - 90 of SEQ ID NO:1.

The open reading frame of the sequence reported at SEQ ID NO:3 predicts a polypeptide of 318 amino acids (identified as residues Met75- Trp392 of SEQ ID NO:4) with the calculated molecular weight of 35,049 and theoretical isoelectric point (pI) = 4.83. This closely approximates the experimentally estimated molecular weight and isoelectric point of P37 PL scramblase purified from erythrocyte membranes. Thus we identify residues 75 - 392 of SEQ ID NO:4 as P37, human plasma membrane PL scramblase and deduce that this same protein is expressed in both erythroid and non-erythroid human cells.

Analysis of the sequence contained in P37 (identified as residues 75 - 392 of SEQ ID NO:4) predicts a membrane protein with a cytoplasmic N-terminus and a single transmembrane helix formed by residues 365 - 383, with residues 384 - 392 projecting from the cell surface and residues 75 - 364 internal to the cell. A site for phosphorylation by protein kinase C or other serine/threonine protein kinase is predicted at P37 Thr residue 235 (all residue numbers for P37 given according to sequence identified at SEQ ID NO:4). It has been observed that activity of PL scramblase is reduced in cells depleted of ATP (Martin, et al., *J. Biol. Chem.* 270:10468-10474, 1995), and that inhibitors of Ca<sup>2+</sup>/calmodulin kinases reduce platelet plasma membrane microparticle formation (Wiedmer, et al., *Blood* 78:2880-2886, 1991), which is consistent with the regulation of the activity of PL scramblase by a phosphorylation of P37 mediated by one or more intracellular protein kinases.

(1) APPLICANT:

- (A) NAME: The Blood Center of Southeastern Wisconsin, Inc.  
(B) STREET: 8701 Watertown Plank Road  
(C) CITY: Milwaukee  
(D) STATE: Wisconsin  
(E) COUNTRY: United States of America  
(F) POSTAL CODE: 53226  
(G) TELEPHONE: (414) 456-4402  
(H) TELEFAX: (414) 266-8658

- (ii) TITLE OF INVENTION: A PROTEIN PREPARATION THAT MEDIATES  
Ca<sup>2+</sup>-DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA  
MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

- (iii) NUMBER OF SEQUENCES: 4

- (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Quarles & Brady  
(B) STREET: 411 East Wisconsin Avenue  
(C) CITY: Milwaukee  
(D) STATE: Wisconsin  
(E) COUNTRY: U.S.A.  
(F) ZIP: 53202-4497

- (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DCS  
(D) SOFTWARE: Patent In Release #1.0, Version #1.25

- (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

- (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Baker, Jean C.  
(B) REGISTRATION NUMBER: 35,433  
(C) REFERENCE/DOCKET NUMBER: 390274.90021

- (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (414) 277-5709  
(B) TELEFAX: (414) 271-3552

## (2) INFORMATION FOR SEQ ID NO:1:

- (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 188 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Phe Gln Gly Pro Pro Gly Tyr Ser Gly Tyr Pro Gly Pro Gln Val  
1 5 10 15

Ser Tyr Pro Pro Pro Pro Ala Gly His Ser Gly Pro Gly Pro Ala Gly  
20 25 30

Phe Pro Val Pro Asn Gln Pro Val Tyr Asn Gln Pro Val Tyr Asn Gln  
35 40 45

- 37 -

Pro Val Gly Ala Ala Gly Val Pro Trp Met Pro Ala Pro Gln Pro Pro  
 50 55 60  
 Leu Asn Cys Pro Pro Gly Leu Glu Tyr Leu Ser Gln Ile Asp Gln Ile  
 65 70 75 80  
 Leu Ile His Gln Gln Ile Glu Leu Leu Glu Val Leu Thr Gly Phe Glu  
 85 90 95  
 Thr Asn Asn Lys Tyr Glu Ile Lys Asn Ser Phe Gly Gln Arg Val Tyr  
 100 105 110  
 Phe Ala Ala Glu Asp Thr Asp Cys Cys Thr Arg Asn Cys Cys Gly Pro  
 115 120 125  
 Ser Arg Pro Phe Thr Leu Arg Ile Ile Asp Asn Met Gly Gln Glu Val  
 130 135 140  
 Ile Thr Leu Glu Arg Pro Leu Arg Cys Ser Ser Cys Cys Cys Pro Cys  
 145 150 155 160  
 Cys Leu Gln Glu Ile Glu Ile Gln Ala Pro Pro Gly Val Pro Ile Gly  
 165 170 175  
 Tyr Val Ile Gln Thr Trp His Pro Cys Leu Pro Lys  
 180 185

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 568 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAGCATTCCA AGGACCTCCA GGATATAGTG GCTACCTCGG GCCCCAGGTC AGCTACCCAC 60  
 CCCCACCAGC CGGCCATTCA GGTCTGGCC CAGCTGGCTT TCCTGTCCCA AATCAGCCAG 120  
 TGTATAATCA GCCAGTATAT AATCAGCCAG TTGGAGCTGC AGGGGTACCA TGGATGCCAG 180  
 CGCCACAGCC TCCATTAAAC TGTCCACCTG GATTAGAATA TTAAAGTCAG ATAGATCAGA 240  
 TACTGATTCA TCAGCAAATT GAACTTCTGG AAGTTTAAAC AGGTTTGGAA ACTAATAACA 300  
 AATATGAAAT TAAGAACAGC TTTGGACAGA GGGTTTACTT TGCAGCGGAA GATACTGATT 360  
 GCTGTACCCG AAATTGCTGT GGGCCATCTA GACCTTTTAC CTTGAGGATT ATTGATAATA 420  
 TGGGTCAAGA AGTCATAACT CTGGAGAGAC CACTAAGATG TAGCAGCTGT TGTGTCCCT 480  
 GCTGCCTTCA GGAGATAGAA ATCCAAGCTC CTCCTGGTGT ACCAATAGGT TATGTTATTC 540  
 AGACTTGGCA CCCATGTCTA CCAAAGTT 568

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1266 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

CGTGTTGTGT ACGTAAGATT CAGGAAACGA AACCAGGAGC CGCGGGTGTT GCGCAAAGG      60
TTACTCCCAG ACCCTTTTCC GGCTGACTTC TGAGAAGGTT GCGCAGCAGC TGTGCCCCGAC      120
AGTCTAGAGG CGCAGAAGAG GAAGCCATCG CCTGGCCCCG GCTCTCTGGA CCTGTCTCG      180
CTCGGGAGCG GAAACAGCGG CAGCCAGAGA ACTGTTTTAA TCATGGACAA AAAAACTCA      240
CAGATGAATG CTTCTCACC GGAAACAAAC TTGCCAGTTG GGTATCCTCC TCAGTATCCA      300
CCGACAGCAT TCCAAGGACC TCCAGGATAT AGTGGCTACC CTGGGCCCCA GGTGAGCTAC      360
CCACCCCCAC CAGCCGGCCA TTCAGGTCTT GGCCAGCTG GCTTTCCTGT CCCAAATCAG      420
CCAGTGTATA ATCAGCCAGT ATATAATCAG CCAGTTGGAG CTGCAGGGGT ACCATGGATG      480
CCAGCGCCAC AGCCTCCATT AAAGTGTCCA CTGGATTAG AATATTTAAG TCAGATAGAT      540
CAGATACTGA TTCATCAGCA AATTGAACTT CTGGAAGTTT TAACAGGTTT TGAAACTAAT      600
AACAAATATG AAATTAAGAA CAGCTTTGGA CAGAGGTTT ACTTTGCAGC GGAAGATACT      660
GATTGCTGTA CCCGAAATTG CTGTGGGCCA TCTAGACCTT TTACCTTGAG GATTATTGAT      720
AATATGGGTC AAGAAGTCAT AACTCTGGAG AGACCACTAA GATGTAGCAG CTGTTGTTGT      780
CCCTGCTGCC TTCAGGAGAT AGAAATCCAA GCTCCTCTG GTGTACCAAT AGGTTATGTT      840
ATTGAGACTT GGCACCCATG TCTACCAAAG TTTACAATTC AAAATGAGAA AAGAGAGGAT      900
GTACTAAAAA TAAGTGGTCC ATGTGTTGTG TGCAGCTGTT GTGGAGATGT TGATTTTGAG      960
ATTAAATCTC TTGATGAACA GTGTGTGGTT GGCAAAATTT CCAAGCACTG GACTGGAATT      1020
TTGAGAGAGG CATTTACAGA CGCTGATAAC TTTGGAATCC AGTTCCTTT AGACCTTGAT      1080
GTTAAATGA AAGCTGTAAT GATTGGTGCC TGTTTCCTCA TTGACTTCAT GTTTTTTGAA      1140
AGCACTGGCA GCCAGGAACA AAAATCAGGA GTGTGGTAGT GGATTAGTGA AAGTCTCCTC      1200
AGGAAATCTG AAGTCTGTAT ATTGATTGAG ACTATCTAAA CTCATACCTG TATGAATTAA      1260
GCTGTA

```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 422 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Arg Val Val Tyr Val Arg Phe Arg Lys Arg Asn Gln Glu Pro Arg Val
 1           5           10           15
Leu Ala Gln Arg Leu Leu Pro Asp Pro Phe Pro Ala Asp Phe Xaa Glu
          20          25          30
Gly Cys Ala Ala Ala Val Pro Asp Ser Leu Glu Ala Gln Lys Arg Lys
          35          40          45

```



Pro Ser Pro Gly Pro Gly Ser Leu Asp Leu Val Ser Leu Gly Ser Gly  
 50 55 60  
 Asn Ser Gly Ser Gln Arg Thr Val Leu Ile Met Asp Lys Gln Asn Ser  
 65 70 75 80  
 Gln Met Asn Ala Ser His Pro Glu Thr Asn Leu Pro Val Gly Tyr Pro  
 85 90 95  
 Pro Gln Tyr Pro Pro Thr Ala Phe Gln Gly Pro Pro Gly Tyr Ser Gly  
 100 105 110  
 Tyr Pro Gly Pro Gln Val Ser Tyr Pro Pro Pro Pro Ala Gly His Ser  
 115 120 125  
 Gly Pro Gly Pro Ala Gly Phe Pro Val Pro Asn Gln Pro Val Tyr Asn  
 130 135 140  
 Gln Pro Val Tyr Asn Gln Pro Val Gly Ala Ala Gly Val Pro Trp Met  
 145 150 155 160  
 Pro Ala Pro Gln Pro Pro Leu Asn Cys Pro Pro Gly Leu Glu Tyr Leu  
 165 170 175  
 Ser Gln Ile Asp Gln Ile Leu Ile His Gln Gln Ile Glu Leu Leu Glu  
 180 185 190  
 Val Leu Thr Gly Phe Glu Thr Asn Asn Lys Tyr Glu Ile Lys Asn Ser  
 195 200 205  
 Phe Gly Gln Arg Val Tyr Phe Ala Ala Glu Asp Thr Asp Cys Cys Thr  
 210 215 220  
 Arg Asn Cys Cys Gly Pro Ser Arg Pro Phe Thr Leu Arg Ile Ile Asp  
 225 230 235 240  
 Asn Met Gly Gln Glu Val Ile Thr Leu Glu Arg Pro Leu Arg Cys Ser  
 245 250 255  
 Ser Cys Cys Cys Pro Cys Cys Leu Gln Glu Ile Glu Ile Gln Ala Pro  
 260 265 270  
 Pro Gly Val Pro Ile Gly Tyr Val Ile Gln Thr Trp His Pro Cys Leu  
 275 280 285  
 Pro Lys Phe Thr Ile Gln Asn Glu Lys Arg Glu Asp Val Leu Lys Ile  
 290 295 300  
 — Ser Gly Pro Cys Val Val Cys Ser Cys Cys Gly Asp Val Asp Phe Glu  
 305 310 315 320  
 Ile Lys Ser Leu Asp Glu Gln Cys Val Val Gly Lys Ile Ser Lys His  
 325 330 335  
 Trp Thr Gly Ile Leu Arg Glu Ala Phe Thr Asp Ala Asp Asn Phe Gly  
 340 345 350  
 Ile Gln Phe Pro Leu Asp Leu Asp Val Lys Met Lys Ala Val Met Ile  
 355 360 365  
 Gly Ala Cys Phe Leu Ile Asp Phe Met Phe Phe Glu Ser Thr Gly Ser  
 370 375 380  
 Gln Glu Gln Lys Ser Gly Val Trp Xaa Trp Ile Ser Glu Ser Leu Leu  
 385 390 395 400  
 Arg Lys Ser Glu Val Cys Ile Leu Ile Glu Thr Ile Xaa Thr His Thr  
 405 410 415  
 Cys Met Asn Xaa Ala Val  
 420

## CLAIMS

## We claim:

1. A preparation of a phospholipid scramblase, wherein the protein is approximately 35-37 kD as measured on a 12.5% SDS-polyacrylamide gel under reducing conditions.
2. The preparation of claim 1, wherein the phospholipid scramblase comprises residues 58 - 90 of SEQ ID NO:1.
3. The preparation of claim 1, wherein the phospholipid scramblase comprises residues 1 - 188 of SEQ ID NO:1.
4. The preparation of claim 1, wherein the phospholipid scramblase comprises residues 75-392 of SEQ ID NO:4.
5. The preparation of claim 1 wherein the protein is isolated from erythrocyte membranes.
6. The preparation of claim 1 wherein the protein is produced by cells genetically modified so as to express the protein of claim 1.
7. The preparation of claim 1 wherein the protein is produced by an organism selected from the group consisting of bacteria, insect cells, or yeast.
8. A recombinant DNA sequence encoding the protein of claim 1.
9. The sequence of claim 8, comprising SEQ ID NO:3.
10. The sequence of claim 8, comprising nucleotides 223-1176 of SEQ ID NO:3.
11. The sequence of claim 8, comprising SEQ ID NO:2 and wherein the sequence is part of a protein expression vector.

12. An inhibitor of phospholipid scramblase activity of the protein of claim 1.

13. The inhibitor of claim 12 wherein the inhibitor is an antisense nucleotide derived from a DNA sequence encoding P37.

14. The inhibitor of claim 12 wherein the inhibitor is an antisense nucleotide derived from a DNA sequence identified at SEQ ID NO:3.

15. The inhibitor of claim 12 wherein the inhibitor is a monoclonal antibody against P37.

16. The inhibitor of claim 12 wherein the inhibitor is a monoclonal antibody against peptide sequence identified as residues 385-392 of SEQ ID NO:4.

17. The inhibitor of claim 15 wherein the monoclonal antibody is selected from the group consisting of recombinantly produced intact IgG, Ig Fab fragment, recombinant Ig Fv or recombinant single chain Ig Fv fragment ScFv.

18. The inhibitor of claim 12 wherein the inhibitor is a peptide containing an amino acid sequence derived from P37, wherein the peptide is a competitive inhibitor of P37 phospholipid scramblase activity.

19. The inhibitor of claim 18 wherein the inhibitor is a peptide that binds to plasma membrane phosphatidylserine.

20. The inhibitor of claim 18 wherein the inhibitor is a peptide that prevents binding of  $Ca^{2+}$  to P37.

21. The inhibitor of claim 18 wherein the inhibitor prevents phosphorylation of P37 by a cellular protein kinase.

22. The inhibitor of claim 21 containing the peptide sequence Thr-Leu-Arg or is a peptidomimetic structural analogue of the tripeptide Thr-Leu-Arg.

23. A method for preventing the surface exposure of  
5 plasma membrane phosphatidylserine, phosphatidylethanolamine and cardiolipin on the surface of *in vitro* stored platelets or red blood cells comprising the step of adding an inhibitor of P37 to the stored platelets or red blood cells.

24. The method of claim 23 wherein the inhibitor is a  
10 nucleotide sequence.

25. The method of claim 23 wherein the inhibitor is a monoclonal antibody against P37.

26. The method of claim 23 wherein the inhibitor is a peptide containing an amino acid sequence derived from P37,  
15 wherein the peptide is an antagonist of P37 phospholipid scramblase activity.

27. The method of claim 26 wherein the inhibitor is a peptide that binds to plasma membrane phosphatidylserine.

28. The method of claim 26 wherein the inhibitor is a  
20 peptide that prevents binding of  $Ca^{2+}$  to P37.

29. The method of claim 26 wherein the inhibitor is a peptide or peptidomimetic peptide analogue that prevents phosphorylation of P37 by a cellular kinase.

30. A method for prolonging graft survival of  
25 transplanted organs and grafts comprising the step of adding an inhibitor of P37 phospholipid scramblase activity to an organ perfusate during *in vitro* organ storage.

31. A method for preventing the procoagulant properties of erythrocytes in sickle cell disease comprising the step of  
30 inhibiting erythrocyte P37 in a sickle cell patient.

32. A method for prolonging the *in vivo* survival of circulating blood cells comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the circulating blood cells by exposing the blood cells to an inhibitor of plasma membrane P37.

33. A method for treating autoimmune, thrombotic, thromboembolic, and inflammatory diseases comprising the step of treating a patient with an inhibitor of the phospholipid scramblase activity of P37.

34. The method of claim 33, wherein the disease is selected from disseminated intravascular coagulation, vascular thrombosis, fibrin generation during cardiopulmonary bypass procedures, rheumatoid arthritis, systemic lupus erythematosus, thrombotic thrombocytopenic purpura, heparin-associated thrombosis, and organ transplant rejection.

35. A method for diagnosing individuals with reduced or elevated capacity for platelet-promoted or erythrocyte-promoted fibrin clot activity comprising the step of quantitating the level of cellular expression of P37 in platelets and erythrocytes in a whole blood sample obtained from the patient mixed with acid-citrate-dextrose, EDTA, heparin, or other suitable anticoagulant.

36. The method of claim 35 wherein quantitation is performed by immunoblotting using an antibody to P37.

37. The method of claim 35 wherein quantitation is performed by ELISA assay using an antibody to P37.

38. The method of claim 35 wherein quantitation is performed by fluorescence-activated flow cytometry using an antibody to P37.

39. The method of claim 38 wherein quantitation is performed using a monoclonal antibody reactive with the peptide sequence corresponding to residues 385-392 of SEQ ID NO:4.

40. The method of claim 35 wherein quantitation is  
5 performed using oligonucleotides derived from P37 cDNA and the polymerase chain reaction.

41. The method of claim 35 wherein quantitation is performed by isolating P37 from a patient venous blood sample, by first isolating the platelets and erythrocytes by  
10 differential centrifugation, and then measuring the amount of P37 present either per cell or per unit of total cell protein, and comparing the measurement with control sample.

42. A genetically engineered cell for transplantation into a human or animal wherein the cell does not express PL  
15 scramblase at native levels of expression.

43. The cell of claim 42 further comprising a nucleotide molecule which is expressed by the cell and which codes for protein inhibiting the activity of PL scramblase.

44. The cell of claim 42 wherein the promoter of the PL  
20 scramblase gene is altered to either increase or decrease the expression of the gene.

45. The cell of claim 42 selected from the group consisting of endothelial cells, fibroblasts, epithelial cells, skeletal cells, cardiac and smooth muscle cells,  
25 hepatocytes, pancreatic islet cells, bone marrow cells, astrocytes, and Schwann cells

46. A prosthesis for implantation in an animal or human having cells of claim 42 attached thereto.

47. The prosthesis of claim 46, wherein the prosthesis is  
30 a vascular graft.

1/3

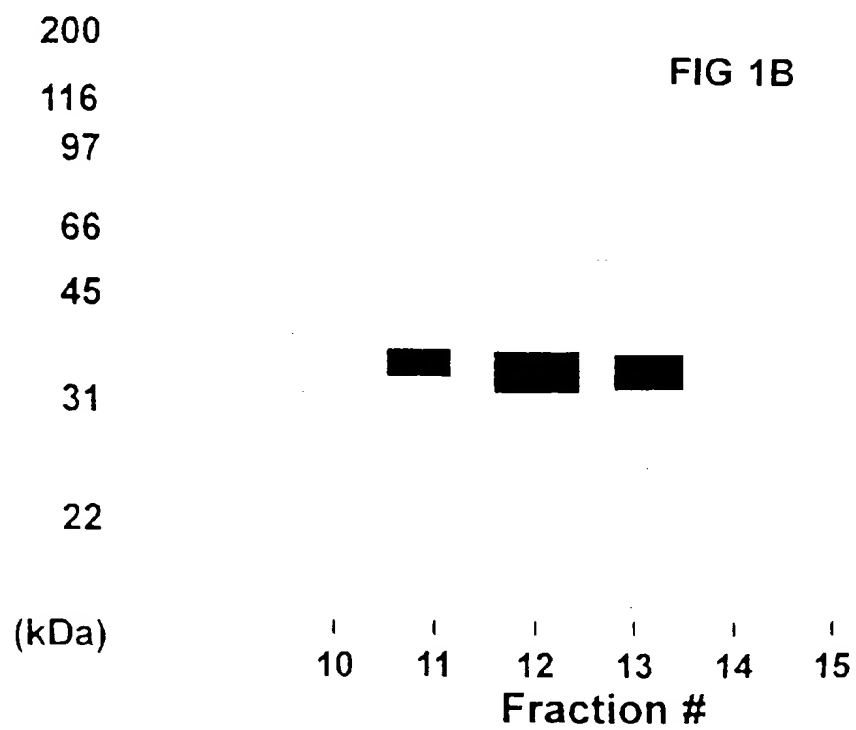
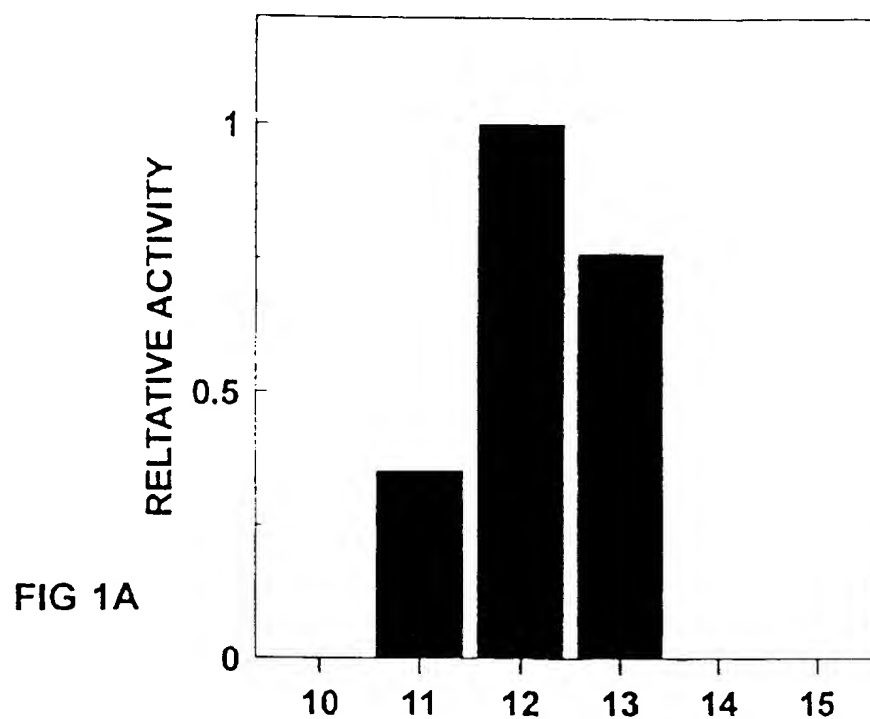


FIG 2

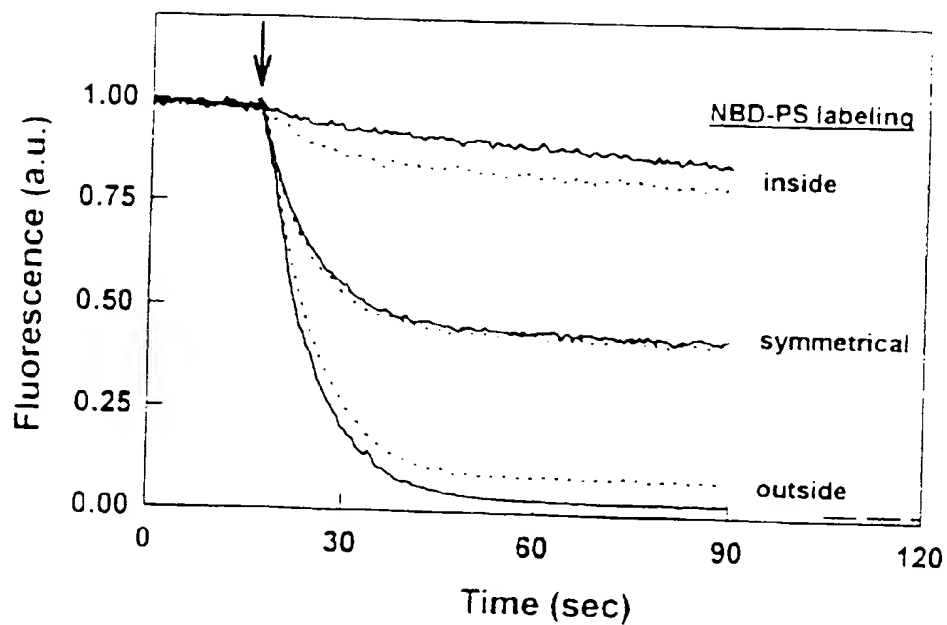
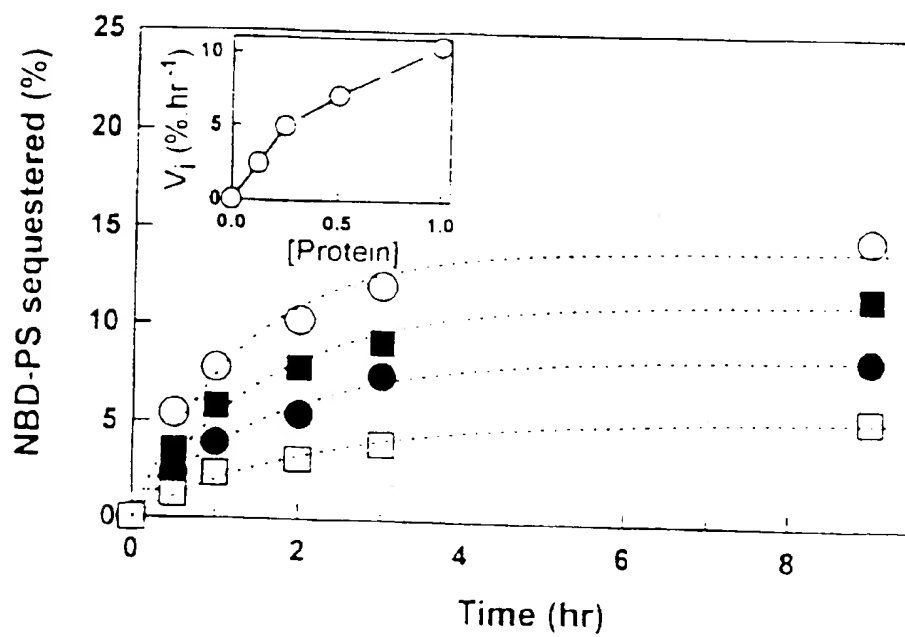
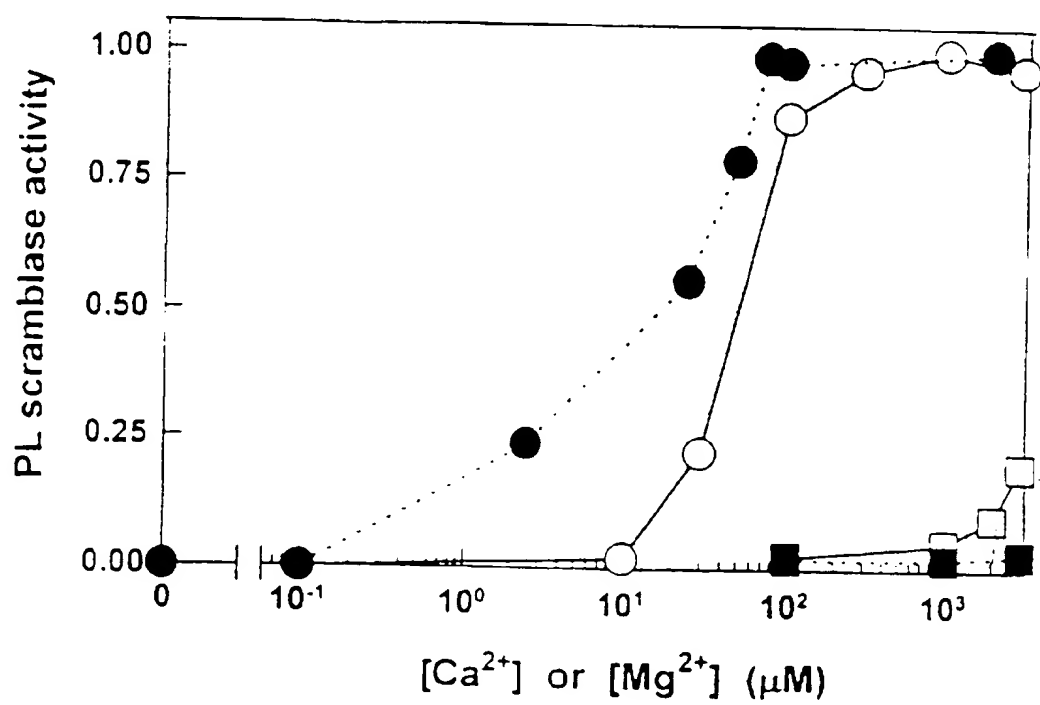


FIG 3





**FIG 4**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/05198

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/573; C12N 9/00; C07K 16/00; C07H 21/04

US CL : 435/6, 7.4, 13, 183; 530/388.15; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.4, 13, 15, 69.1, 183; 530/350, 388.15, 388.26; 536/23.5  
424/9.1, 422, 146.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, CJACS  
Sequence Databases (GENBANK, EMBL, PIR, SWISSPROT, EST, GENESEQ)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y,P	Database, MPSRCH, SMITH-WATERMAN algorithm. Databases n-geneSEQ26 and EST-STS. May 1997, relevant hits, see entire document.	1-11 ---- 12-31, 33-47
X,P ---- Y,P	BASSE et al. Isolation of an Erythrocyte Membrane Protein that Mediates Ca <sup>2+</sup> -dependent Transbilayer Movement of Phospholipid. J. Biol. Chem. 19 July 1996, Vol. 271, No. 29, pages 17205-17210. See entire document.	1-11 ---- 12-31, 33-47
X,P ---- Y,P	COMFURIUS et al. Reconstitution of Phospholipid Scramblase Activity from Human Blood Platelets. Biochem. 18 June 1996, Vol. 35, No. 24, pages 7631-7634. See entire document.	1-11 ---- 12-31, 33-47

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	* T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

26 MAY 1997

Date of mailing of the international search report

11 JUL 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DANIEL MYTELKA

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/US97/05198

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KOJIMA et al. Production and Characterization of Transformed B-Lymphocytes Expressing the Membrane Defect of Scott Syndrome. J. Clin. Invest. December 1994, Vol. 94, pages 2237-2244. See entire document.	1-29
Y	SMEETS et al. Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes. Biochem. Biophys. Acta. 02 November 1994, Vol. 1195, pages 281-286. See entire document.	1-29
Y	VERHOVEN et al. Mechanisms of Phosphatidylserine Exposure, A Phagocyte Recognition Signal, on Apoptotic T Lymphocytes. J. Exp. Med. November 1995, Vol. 182, pages 1597-1601. See entire document.	12-30, 33-34
Y	DEVAUX et al. Maintenance and consequences of membrane phospholipid asymmetry. Chem. Phys. Lipids. 06 September 1994, Vol. 73, pages 107-120. See entire document.	23-29
Y	Database MEDLINE on STN, No. 90158550, ZWAAL et al. Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled cells; mechanisms and physiological significance. Mol. Cell. Biochem. 23 November-23 December 1989, Vol. 1-2, pages 23-31. Abstract.	31

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/05198

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-31 and 33-47
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/05198

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-11, drawn to phospholipid scramblase protein and DNA encoding same.

Group II, claim(s) 12-22, drawn to inhibitors of scramblasas.

Group III, claim(s) 23-29, drawn to uses of inhibitors to prevent surface exposure of phospholipids in stored red blood cells or platelets.

Group IV, claim(s) 30, drawn to uses of inhibitors to prolong graft survival.

Group V, claim(s) 31, drawn to uses of inhibitors to prevent coagulation of cells in a sickle cell patient.

Group VI, claim(s) 32, drawn to uses of inhibitors to prolong red blood cell lifespan in vivo.

Group VII, claim(s) 33-34, drawn to uses of inhibitors to treat autoimmune diseases.

Group VIII, claim(s) 35-41, drawn to methods of diagnosing patients with abnormal capacities for clot formation.

Group IX, claim(s) 42-47, drawn to methods of engineering cells for transplantation.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I and Group II are drawn to separate products that do not necessarily have anything in common, either structurally or functionally. For example, the scramblasas of Group I are  $\text{Ca}^{++}$  dependent, so they would be inhibited by any compound that chelates divalent cations, such as EDTA or citric acid. Groups III-VII are drawn to five separate uses of the inhibitors. Group VIII includes methods of diagnosis that do not necessarily use the first named product (they might instead use antibodies or coagulation tests). Group IX includes methods of engineering cells that do not necessarily involve any direct changes in the compounds of group I, such as the addition of nucleic acids coding for an inhibitor.

